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14. **ABSTRACT**
We are investigating the role of inflammation in the death of the dopamine (DA) neuron in the substantia nigra of MPTP-treated mice. Studies in our model over the course of this program project show that microglia are the source of superoxide and nitric oxide through up-regulation of the microglial enzymes NADPH oxidase and inducible nitric oxide synthase (iNOS). We show that activation of microglia *in vivo* occurs as early as 24 hours after MPTP administration at which time the presence of superoxide in the SNpc after MPTP is noted using hydroethidium histochemistry. M40401, a nonpeptidyl SOD mimetic with catalytic activity equal to or greater than native MnSOD decreased the presence of superoxide and attenuated microglial activation in the SNpc of our mice. Minocycline, a second generation antibiotic, inhibited iNOS up-regulation and also reduced microglial activation. Further, we demonstrate that cyclooxygenase-2 is a part of the inflammatory response in MPTP-treated mice. Our neuronal/microglial cultures now that they are up and running, should give a more exact timeframe of microglial activation in relation to DA neuron death in the MPTP mouse model of PD. Furthermore, we identify components of microglia that may be therapeutic targets.

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Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder characterized behaviorally by resting tremor, rigidity, akinesia/bradykinesia and postural instability (Fahn and Przedborski, 2000). Neurons associated with the behavioral component of PD are mainly, though not exclusively, the dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) which degenerate and die during the course of the disease (Fahn and Przedborski, 2000). Although we do not know the etiology of this disorder, our studies using the MPTP mouse model of PD and tissues from PD brains indicate 1) that the superoxide radical and nitric oxide are implicated in PD (Przedborski et al, 1992; Przedborski et al, 1996; Liberatore et al, 1999); 2) that there is a greater loss of dopaminergic terminals in the striatum than the loss of dopaminergic neurons in the SNpc (Jackson-Lewis et al, 1995); 3) that there is an inflammatory component to PD that may be the cause of its progressive nature (Liberatore et al, 1999; Wu et al, 2002); and 4) that there is an up-regulation of certain cytokines in the SNpc of both MPTP-treated mice and tissues from PD brains (Teismann and Przedborski, personal communication). The oxidative stress hypothesis of PD seems to encompasses all of these findings. While etiological factors may ignite PD's neurodegenerative process, additional factors must participate in the progression of this debilitating disorder as DA neurons continue to die, albeit at a lower rate for many years as shown in recent evidence from human brains of individuals who had injested MPTP (Langston et al, 1999). Since our goal is to elucidate the pathogenic factors that contribute to the progression of PD, we revisited the pathological picture of PD in search of abnormalities that might shed some light onto these additional factors. Aside from the severe loss of DA neurons in the SNpc, there is also present a marked glial response in both the PD brain and the MPTP-treated brain (Langston et al, 1999). Interestingly, both astrocytes and microglia, the two main components of the microglial response, are up-regulated in both situations (McGeer et al, 1988; Forno et al, 1992; Czlonkowska et al, 1996; Oppenheimer et al, 1997; Banati et al, 1998; Mizra et al, 2000), however, the magnitude of their responses is quite different. Although both are thought to support DA neuron survival (Abbott, 1988), whereas astrocytes are thought to provide trophic support to the DA neuron (Abbott, 1988), microglia are thought to represent a two-edged sword in that they can be both supportive and deleterious (Banati et al, 1993). The astrocytic response, both in number and in immunoreactivity, is, at best, mild and in only a few instances, has this response been dramatic (Forno et al, 1992; Mirza et al, 2000). In contrast, the activation of microglia has been consistently strongest in the SNpc the area of the brain most affected by the neurodegenerative process (Vila et al, 2001) thus, possibly counter-productive to the survival of the DA neuron due, in part, to their production of the superoxide radical, nitric oxide and a host of deleterious cytokines (Banati, 1993). Merely because the substantia nigra (SN) contains significantly more microglia than other brain areas (Smeyne et al, 2005), by virtue of this fact, DA neurons are in an environment that can increase their vulnerability to damage or worst, to death in the event of a toxic insult or injury. Activated microglia are also found in close proximity to any remaining dopaminergic neurons (McGeer et al, 1988), around which they sometimes cluster to produce what resembles neurophagia. Microglial activation and neurophagia are indicative of an active ongoing process of cell death (Langston et al, 1999) which is

consistent with the progressive nature of PD. However, few data exist on the temporal relationship between dopamine neuron death and microglial activation. To this end, we have proposed to use the MPTP mouse model to sort out the process of gliosis as it relates to the time course of and the events of microglial activation in SNpc dopaminergic neuron death. Aside from the up-regulation of microglia early on in the MPTP neurotoxic process (Liberatore et al, 1999), we have noted that microglia systematically produce a number of pro-inflammatory compounds (Teismann and Przedborski, personal communication), which we think may be some of the factors which participate in the progressive nature of DA neuron death in PD.

Microglial cells are resident macrophage cells in the brain that have the ability to react promptly in response to brain injury and to subtle changes in the microenvironment surrounding their charges, which are, in the SNpc, the DA neurons (Kreutzberg, 1996). Normally, brain microglial cells are kept in a resting state in which they are barely visible and very few, if any, ramified processes can be detected. In a pathological situation, microglia quickly become up-regulated, proliferate, become hypertrophic, increase in size and produce a number of marker molecules that are either pro- or anti-inflammatory in nature (Banati et al, 1993; Kreutzberg, 1996). Pro-inflammatory compounds include the superoxide radical, nitric oxide (NO), prostaglandin E_2 (PGE_2), excitatory amino acids, and pro-inflammaroty cytokines such as interleukin-1-beta (IL-1-ß). Since activated microglia have been found in the SNpc of PD brains and in the MPTP mouse model of PD, and can produce such damaging molecules, it is important to discern their mechanism of activation in PD and in the MPTP mouse model of PD as well as to continue our efforts to identify compounds that exhibit neuroprotective qualities in the SNpc. Since this is a Program Project with several components, we will concern ourselves with the Project Core (Core A and Core B) and Project .1 (Przedborski).

Body of the Research.

The overall goal of this research project is the study of the pathogenesis of PD using the oxidative stress hypothesis of PD as its basis. Part of our research has led us to note that microglia are activated during the MPTP neurotoxic process. Furthermore, this fact has been shown to be true for PD brains and for the brains of individuals who had injected themselves with MPTP. Thus, we reasoned that microglial activation must be an integral part of the neurodegenerative process so characteristic of PD and the MPTP model of PD. We further thought that microglial activation may be the reason for the progressive nature of PD and that elucidating the mechanisms of microglial activation would be important to our understanding of the progressive nature of the disease as this may help us to identify possible targets for treatments which may improve the symptomology of or stop its progression. We also reasoned that if our MPTP model gave us clues to microglial activation here, then we might be able to apply what we find to the human condition. In this respect, in **Specific Aim I**, we propose to determine the role of microglial activation in the MPTP neurotoxic process. Our plan is to administer different doses of MPTP to mice pretreated with different doses of minocycline, a drug known to block microglial activation independent of its antimicrobial effects, then assess neuroprotection in the SNpc using HPLC, immunstaining and Western blot analyses. We also plan to use primary mixed neuronal/microglial cultures to study more definitively the role of

activated microglia in MPTP-mediated DA neuronal death and the contribution of proinflammatory factors. We will also assess, by pharmacological intervention, the beneficial effects of inhibiting such proinflammatory factors. In **Specific Aim II**, we propose to define the role of NADPH oxidase in the MPTP neurotoxic process at different time points in the MPTP mouse model of PD using both normal mice and mice that are deficient in NADPH oxidase We will assess neuroprotection and process of microglial activation. In **Specific Aim III**, we propose to assess the neuroprotective effect of M40401 on SNpc DA neuron death in the MPTP mouse model of PD. M40401 is a manganese-based superoxide dismutase (SOD) mimetic that readily crosses the blood-brain barrier and prevents the production of the superoxide radical. We will assess the protective effect of M40401 in brain tissues known to be damaged by MPTP at different time points and at different doses using the same methodology as for Specific Aim I. Finally, in **Specific Aim IV,** we plan to examine the contribution of prostaglandin PGE_2 to the death of the SNpc DA neurons by assessing the roles of the PGE_2-synthesizing enzymes cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2 in the MPTP neurotoxic process in different brain regions and at different time points using different doses of the toxin. We also plan to use COX-1 and COX-2 deficient mice in these studies. These planned specific aims should provide valuable information about the mechanisms involved in the inflammatory response related to the MPTP neurotoxic process and to PD as well as identify targets for therapeutic intervention.

Key Research Accomplishments

Core A.

Core A continues to be the administrative arm of this Parkinson's Disease Research Program. It role is to provide the centralized scientific leadership so necessary for this program to proceed and to succeed. Core A reviews all of the findings of this project, discusses them with the researchers and provides guidance in the reporting of results in publications. At this point, because of the excellent guidance afforded by Core A, only the cell culture part of this project remains to be done.

Core B.

Core B is the centralized MPTP facility for this project. It is located within the Animal Care Facility on the 19th floor of the College of Physicians and Surgeons here at Columbia University. The primary role of Core B is to support the research activities under this project. To this end, Core B represents the first leg of this research project and the work here encompasses four specific aims all geared toward supplying the needed MPTP-treated mice for this program project as well as the training of our researchers in the safe use of MPTP. Procedures have been worked out to ensure both. All mice are ordered and received in the Animal Care Facility. Mice are housed by animal care technicians of this facility, then placed outside of the MPTP Facility. Core B is informed of their arrival by telephone and a member of the Core B staff puts the housed mice inside the MPTP room and logs them in. For reception of genetically engineered mice from other institutions, a health report is required. In house genetically engineered mice are bred in the barrier facility of the animal care facility and at the age of one month are transferred to the MPTP Facility and logged in. All animals acclimate for at least one week prior to any injection schedule. Experiments for the tissues necessary for the Program Project are discussed and injection regimens are

scheduled with Dr. V. Jackson-Lewis. All injections are performed by Dr. Jackson-Lewis. Samples are then collected according to the scheduled experiment, and are either used here at Columbia or sent as per the scheduled experiments to the individual who needs them. No glitches in shipping samples have occurred thus far. To insure that samples are prepared properly, quality controls are run frequently with experimental samples on the HPLC. As a result of this policy, our quality control results indicate that our results are highly reproducible and that intra-group variation is less than 5%. All research fellows who handle the MPTP-treated mice are also required to read our paper regarding the safe handling of MPTP mice and samples (Przedborski et al, 2001) and are trained in the handling of same by Dr. Jackson-Lewis.

Specific Aim I.

In Vivo Experiments.

The in vivo work using minocycline as a neuroprotective agent in the MPTP mouse has been done and has been published in the Journal of Neuroscience in 2002 under the title **Blockade of microglial activation is neuroprotective in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson disease.** J Neuroscience 22: 1763-1771, The important findings in this study were that minocycline, a second generation tetracycline antibiotic, 1) protects SNpc DA neurons against the damaging effects of MPTP; 2) decreases MPTP-mediated nitrotyrosine formation; 3) inhibits microglial activation; 4) prevents the production of microglial-derived pro-inflammatory cytokines; and 5) confers resistance beyond iNOS (inducible nitric oxide synthase) ablation.

Cell Culture Experiments for Specific Aim I.

Our cell culture facility is now up and running. The microglial cultures have now been established and are stable. One of our research fellows is now establishing neuronal/glial cultures so as to continue our studies in Specific Aim I. Co-cultures will be treated with MPTP or its active metabolite, MPP+, in the absence of and in the presence of varying concentrations of minocycline in order to sort out the mechanism and events of microglial activation as it relates to DA neuron death.

Specific Aim II.

The proposed studies for Specific Aim II has been completed and our results have been published in the Proceedings of the National Academy of Science in 2003 under the title **NADPH oxidase mediates oxidative stress in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of Parkinson's disease** (PNAS 100: 6145-6150). Findings in this study show that 1) NADPH oxidase is induced in the ventral midbrain of MPTP-treated mice; 2) NADPH oxidase is expressed in microglia in the SNpc following MPTP administration to mice; 3) the lack of the gp91 subunit of NADPH oxidase attenuates MPTP-induced reactive oxygen species (ROS) production in the SNpc of MPTP-treated mice as demonstrated by hydroethidium fluorescence; 4) gp91 deficient MPTP-treated mice exhibit less damage to the tyrosine hydroxylase (TH)-positive neurons in the SNpc than their non-deficient counterparts; 5) gp91 deficient mice show significantly less protein carbonyl content in the ventral midbrain of MPTP-treated mice that their wild-type littermates; 6) MPTP toxicity in the SNpc is attenuated by the delivery of extracellular SOD.

Specific Aim III

Specific Aim III proposes to assess the neuroprotective effects of M40401, a superoxide dismutase (SOD) mimetic compound, in MPTP-treated mice. Our hypothesis here is that if we can block the production of the superoxide radical, we can possibly attenuate SNpc DA neuron death. In our animal studies with M40401, we noted that 1) M40401 has a significant neuroprotective effect on the SNpc TH-positive neurons against the damaging effects of MPTP; 2) this effect is not extended to DA terminals in the MPTP-treated striatum even with a lower dose of MPTP; 3) there is a significant decrease in the microglial response to MPTP on MAC-1 immunostaining; 4) there is a significant attenuation of damaged proteins as assessed by carbonyl content in those brain areas known to be affected by MPTP; 5) these effects were due to a significant (@ 50%) reduction in the production of the superoxide radical as demonstrated by hydroethidium histochemistry. The manuscript representing this work presently entitled **M40401, a SOD mimetic, attenuates DA neuron death in the MPTP mouse model of Parkinson's disease** has been written and is now being edited by its authors.

Specific Aim IV.

Epidemiological studies suggest that inflammation increases the risk of developing some kind of neurodegenerative condition. Along with microglial activation, other proinflammatory compounds and cytokines such as cyclooxygenase and prostaniods (prostaglandin E_2 (PGE_2) up-regulate and are reported to be integral parts of the inflammatory response. Thus, it is reasonable to think that these factors might also contribute to the degeneration of SNpc DA neuron in PD and in the MPTP model of PD. The cyclooxygenase (COX) enzymes, COX-1 and COX-2 convert arachidonic acid to prostaglandin H_2 (PGH_2), the precurser of the prostaglandin E_2 (PGE_2) and several other prostanoids (O'Banion, 1999). In our studies using MPTP in COX-1 and COX-2 knockout mice and their wild-type littermates, we demonstrated 1) that the COX-2 enzyme rather than COX-1 is up-regulated in SNpc TH-positive neurons in PD brains and in the ventral midbrain of the MPTP mouse model of PD; 2) that this COX-2 up-regulation in the ventral midbrains of MPTP-treated mice and in PD brains occurs through a Jun kinase (JNK)/c-Jun-dependent mechanism; 3) that COX-2 inhibition and ablation in the MPTP mouse model of PD prevents the formation of dopamine-o-quinone; 4) that COX-2 inhibition and ablation in the MPTP mouse model of PD attenuates TH-positive neuron death in the SNpc of treated mice; 5) that the inhibition of COX-2 may be a possible therapeutic target for drug therapy in PD. This work was completed and is published under the title **Cyclooxygenase-2 is instrumental in Parkinson's disease neurodegeneration** (PNAS 100: 5473-5478, 2003). In our continuing studies of inflammation in PD, we also investigated whether COX-2 was involved in the apoptotic form of death of SNpc DA neurons induced by a sub-acute regimen of MPTP intoxication (30 mg/kg per day for 5 consecutive days). At the peak of apoptotic DA cell death induced by this regimen of intoxication (day 4 after the last MPTP injection), ablation of COX-2 significantly attenuated the number of SNpc apoptotic cells (40% less of SNpc apoptotic cells). To determine whether the reduction of apoptotic cell death resulted in an increased survival of DA cells in this model, we then counted by stereology the number of SNpc tyrosine-hydroxylase positive cells in COX-2-

deficient mice, compared to their wild-type littermates. At day 21 after the last MPTP injection, the number of surviving SNpc DA cells was significantly higher in COX-2-deficient mice (75% of saline-injected animals) than in their wild-type littermates (50% of saline injected animals). Overall, our findings indicate that COX-2 plays a role in both forms of DA neuron death elicited by MPTP, be it necrotic or apoptotic elicited by specific MPTP regimens. The second part of this work is still ongoing and has not yet been published.

Reportable Outcomes for 2004-2005.

Core B.

This year, Dr. Jackson-Lewis was consulted by several outside institutions on the safe handling and use of MPTP. She also wrote protocols for MPTP use for several of our former research fellows who are now researchers at other institutions. Also, she is now working with researchers from Woods Hole to sort out their methodological problems with tissues from MPTP-treated mice. On annual inspection of the MPTP Facility by the university's Institutional Animal Care and Use Committee, no deficiencies have been found in the MPTP facility. Furthermore, our MPTP facility has been visited twice this year by other university investigators to see how an independent facility operates.

Cell Culture Experiments for Specific Aim I.

Our cell culture facility is now up and running. We have secured all of the necessary equipment (hoods, fluorescent microscope, inverted microscope, incubators, etc.) for this part of Specific Aim I. The microglial cultures have now been established and are stable. These cells when treated with lipopolysaccharide can produce reactive oxygen species which we can be demonstrated by fluorescence. With the help of Dr. David Sulzer (DA specialist and a Principal Investigator in our division), one of our research fellows, Dr. Delphine Prou, has now established neuronal cultures grown on microglia. These cultures are now stable and ready to be used in the completion of Specific Aim I. As proposed, neuronal/glial cultures will be treated with varying concentrations of minocycline and MPTP or MPP+ (MPTP active metabolite) in order to sort out the relationship between the DA neuron and microglia in the neurodegenerative process.

Publications (2004-2005) Supported by This Award

Choi, D-K, Pennathur, S, Perier, C, Tieu, K, Teismann, P, Wu, DC, Jackson-Lewis, V, Vila, M, Vonsattel, J-P, Heineke JW, Przedborski, S. **Ablation of the inflammatory enzyme myeloperoxidase mitigates features of Parkinson's disease in mice.** J Neuroscience (2005) 25: 6594-6600.

Perier, C, Tieu, K, Guegan, C, Caspersen, C, Jackson-Lewis, V, Carelli, V, Martinuzzi, A, Hirano, M, Przedborski, S, Vila, M. **Complex I deficiency primes Bax-dependent neuronal apoptosis through mitochondrial oxidative damage.** PNAS (2005) 102: 19126-19131.

Jackson-Lewis, V, Smeyne, RJ. **MPTP and SNpc DA neuronal vulnerability: role of dopamine, superoxide and nitric oxide in neurotoxicity. Minireview.** Neurotoxicity Research (2005) 7: 193-201.

Smeyne, RJ, Jackson-Lewis, V. **The MPTP model of Parkinson's disease.** Molecular Brain Research (2005) 134: 57-66.

Przedborski, Serge. **Pathogenesis of nigral cell death in Parkinson's disease.** Parkinsonism and Related Disorders (2005) 11: S3-S7.

Przedborski, S, Ischiropoulos, H. **Reactive oxygen and nitrogen species: weapons of neuronal destruction in models of Parkinson's disease.** Antioxid Redox Signal (2005) 7: 685-693.

Discussion and Conclusions

The loss of SNpc DA neurons in the brains of PD patients and in the MPTP model of PD is thought to be related to oxidative stress, mitochondrial dysfunction and energy crisis. Neuropathological studies (Langston et al, 1999) indicate that following the acute phase of DA neuron death, neurons continue to die for many years but at a much lower rate. In revisiting the neuropathology of PD, it was noted that aside from the dramatic loss of DA neurons in the SNpc, there is a marked gliosis in the area of the SNpc in PD brains and in the SNpc of MPTP models of PD. This marked gliosis (glial response) occurs in the non-neuronal support systems, the glia, in the SNpc. The question is why does PD take on a progressive nature and how do microglia fit into the picture? It is known that microglia can be supportive of DA neurons as, on one hand, they can produce trophic factors which promote the life of the cell. On the other hand, microglia can produce compounds such as superoxide, tumor necrosis factor, glutamate and arachidonic acid which can be damaging to the DA neurons in the SNpc given the fact that microglial residents here outnumber microglial residents in other brain areas (Smeyne et al, 2005). Thus, microglia put SNpc DA neurons in a precarious microenvironment. Our answer may lie in discerning the temporal relationship between SNpc neuron death and microglial activation. To this end, since MPTP also elicits a microglial response, it is an ideal tool to study this problem. We noted that, in the MPTP mouse model, a strong microglial activation occurs much earlier than that of astrocytes and reaches maximun before the peak of SNpc DA neuron death (Liberatore et al, 1999). From this finding, we concluded that microglial activation must indeed be an integral part of the MPTP neurotoxic process.

Since microglia have been shown to produce a number of proiflammatory compounds, it is necessary to examine microglia in light of these compounds following a toxic insult. In previous publications, we found that following MPTP administration, both the superoxide radical (Przedborski et al, 1992) and nitric oxide (Przedborski et al, 1996; Liberatore et al, 1999) production increase dramatically and localize to activated microglia (Liberatore et al, 1999; Wu et al, 2002). This is in keeping with the oxidative stress hypothesis of PD in that it is thought that superoxide and nitric oxide interact to form peroxynitrite, a compound that damages DNA, proteins, enzymes amino acids and neurotransmitters. Microglia afford a

milieu in which the interaction of these two compounds can take place. Proof of this interaction can be seen in the increased levels of nitrotyrosine in the SNpc of MPTP-treated mice. In Specific Aim I, a decrease in the presence of nitrotyrosine was achieved through the use of minocycline which also attenuated microglial activation through its blockade of the inducible nitric oxide synthase (iNOS) enzyme, the source of nitric oxide. Nitrotyrosine appearance occurred as early as 24 hours after MPTP administration, the same time that microglial activation reaches its peak. Proof that the source of superoxide is also the microglia is our demonstration of superoxide presence in the SNpc using the fluorescent probe hydroethidium (Wu et al, 2002). This effect was also noted at 24 hours after MPTP administration. In Specific Aim II, we show that NADPH oxidase in microglia in the area of the SNpc is up-regulated as early as 24 hours after MPTP administration and that ablation of the gp91 subunit of this enzyme is protective to neurons here (Wu et al, 2003). Associated with this ablation is a decrease in the activation of microglia as well as a decrease in the presence of the superoxide radical. .Thus, our studies define the roles of both superoxide and nitric oxide in the glial response as part of a toxic event and note that their involvement in this response occurs in a time-dependent manner.

Since we have demonstrated that the superoxide radical is an integral part of microglial activation in PD, pharmacological intervention may prove helpful here in abating the death of SNpc DA neurons and possibly the progression of neurodegeneration. To test this possibility, we used M40401, a manganese (Mn) SOD mimetic, in the MPTP mouse model. M40401 posesses a catalytic rate constant that is at least equal to or greater than the native SOD enzymes (Salvemini et al, 1999). Key to its action is the fact that M40401 has a MnII center that is difficult to oxidize and that has no reactivity until it is oxidized to MnIII by protonated superoxide. Once reactive, the MnIII is rapidly reduced back to MnII state by the superoxide anion at diffusion-controlled rates (Salvemini et al, 1999). In the MPTP-treated mouse, M40401 protected SNpc DA neurons to a significant degree although it had little or no effect on DA terminals in the striatum of these animals. It also decreased carbonyl content in the mouse ventral midbrain by preventing oxidation of neuronal proteins and reduced the microglial response to MPTP in this area of the brain. The reduction in microglial activation is most likely due to a decrease in the presence of the superoxide radical as demonstrated by hydroethidium fluorescence.

Expression of cyclooxygenase has emerged as an important determinant of cytotoxicity associated with inflammation (Seibert et al, 1995). The cyclooxygenase (COX) enzyme is the rate-limiting enzyme in the synthesis of prostaglandin E_2 (PGE_2) which facilitates the reaction between the superoxide radical and NO in the formation of peroxynitrite within the DA neuron (Hastings, 1995). Superoxide presence within the dopamine neuron can be the result of DA breakdown as DA is quite unstable or it result from the blockade of complex I of the mitochondrial electron transport chain (Klivenyi et al, 1998). NO may be produced within the DA neuron through the action of neuronal NOS (Jackson-Lewis and Smeyne, 2005). Two isoforms (COX-1 and COX-2) of the COX enzyme exist and their patterns of distribution are somewhat different. The fact that COX-2 co-expresses with iNOS in SNpc microglia from PD brain tissues (Knott et al, 2000) and that its product PGE2 is also elevated here (Rothwell, 1998) led us to investigate the role of COX-2 in the MPTP neurotoxic process. In the mouse model of PD following MPTP administration, COX-2 is

indeed up-regulated in SNpc neurons. Our finding of COX-2 up-regulation in SNpc neurons from PD brains complemented our MPTP findings. Furthermore, we noted that COX-2 deficient mice had significantly more TH-positive neurons in the SNpc than their wild-type counterparts following MPTP administration which suggests that the reaction between superoxide and NO was abated. The administration of CEP11004 to MPTP-treated mice blocks jun kinase activation which, in turn, lessened significantly the up-regulation of the COX-2 enzyme in our model and protected the SNpc DA neurons against the damaging effects of MPTP. We also noted that COX-2 does not participate in the activation of microglia as although COX-2 ablation and inhibition attenuated MPTP-induced SNpc DA neuron death, this approach did not lessen microglial activation (Teismann et al, 2003). Thus, COX-2 because of its up-regulation within the neuron, likely contributes to perturbations within the internal environment of the DA neuron which can lead to damage and even death of the DA neuron in the SNpc in a pathological situation.

It is our conclusion that activated microglia do contribute to the death of SNpc neurons. Their activation is a timely event occuring shortly after a toxic insult and continuing via constant production of superoxide and NO due to the ongoing death of the SNpc DA neurons. We will be able to give a more precise answer here once we have finished the neuronal/microglia cell culture experiments. Regardless of that, it appears that the neurodegenerative process in the SNpc of PD patients and in the MPTP mouse model is a process of cell death that is basically circular in nature.

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Addendum

Publications for this year of the award will be sent by FedEx.

ANTIOXIDANTS & REDOX SIGNALING
Volume 7, Numbers 5 & 6, 2005

Forum Review

Reactive Oxygen and Nitrogen Species: Weapons of Neuronal Destruction in Models of Parkinson's Disease

SERGE PRZEDBORSKI[1] AND HARRY ISCHIROPOULOS[2]

ABSTRACT

Parkinson's disease (PD) is a common neurodegenerative disease whose etiology and pathogenesis remain mainly unknown. To investigate its cause and, more particularly, its mechanism of neuronal death, numerous *in vivo* experimental models have been developed. Currently, both genetic and toxic models of PD are available, but the use of neurotoxins such as 6-hydroxydopamine, paraquat, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, and rotenone are still the most popular means for modeling the destruction of the nigrostriatal dopaminergic neurons seen in PD. These four neurotoxins, although distinct in their intimate cytotoxic mechanisms, kill dopaminergic neurons via a cascade of deleterious events that consistently involves oxidative stress. Herein, we review and compare the molecular mechanisms of 6-hydroxydopamine, paraquat, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, and rotenone, placing the emphasis of our discussion on how reactive oxygen and nitrogen species contribute to the neurotoxic properties of these four molecules. As the reader will discover, to achieve the above stated goal, we had to not only appraise recent findings, but also revisit earlier landmark studies to provide a comprehensive view on this topic. This approach also enabled us to describe how our understanding of the mechanism of actions of certain toxins has evolved over time, which is particularly striking in the case of the quatrogenarian neurotoxin, 6-hydroxydopamine. *Antioxid. Redox Signal.* 7, 685–693.

INTRODUCTION

PARKINSON'S DISEASE (PD) affects ~1% of the population over the age of 50 in the United States alone, and it is the second most frequent neurodegenerative disorder after Alzheimer's disease (15). This common neurodegenerative disorder is essentially a sporadic disease, meaning that it presents itself with no apparent genetic linkage (15). Yet in rare instances, as in several other neurodegenerative diseases (70), PD can be inherited (16). Whether it is sporadic or familial, PD is a slow, progressive disease characterized mainly by resting tremor, slowness of movement (bradykinesia), stiffness (rigidity), and poor balance (postural instability) (25). Most, if not all, of these clinical abnormalities are attributed to the severe loss of the nigrostriatal dopaminergic neurons in the substantia nigra pars compacta (SNpc), which leads to a profound deficit in brain dopamine (15). Another pathological hallmark of PD is the eosinophilic intraneuronal proteinaceous inclusion called the Lewy body (27), whose pathogenic significance remains controversial.

There is no evidence that PD patients must be treated upon emergence of the clinical symptoms. However, at some point, the motor disability becomes so severe that treatment aimed at either replenishing dopaminergic stores in the brain (*e.g.*, levodopa) or stimulating dopamine receptors (*e.g.*, dopamine agonists), or both, is required to maintain the patient's autonomy and quality of life. Several of the approved drugs for PD are quite potent in alleviating symptoms, but their chronic administration often causes serious motor and psychiatric side effects (24).

Regardless of the nature of the etiologic factor that initially provokes neurodegeneration, two major hypotheses regarding

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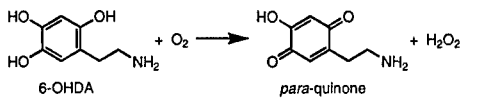
the pathogenesis of the disease have emerged from studies probing the functions of genes implicated in inherited forms of PD and from animal and cellular model systems of PD. One hypothesis postulates that inappropriate aggregation of proteins is instrumental in the death of SNpc dopaminergic neurons, whereas the other, which is the focus of this review, suggests that the offender is oxidative stress, including potentially toxic intermediates of oxidized dopamine. This latter hypothesis posits that the fine-tuned balance between the production and destruction of oxidants is altered in such a way that oxidative damage arises, leading to cellular dysfunction and, ultimately, to cell death. Unquestionably, support for the "oxidative stress hypothesis" of PD comes from descriptive investigations performed on fluids and tissue samples of PD patients (64). However, in our opinion, the most compelling evidence for a role of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the death of SNpc dopaminergic neurons in PD originates, not from human studies, but rather from investigations in animal models of PD generated by various neurotoxins. What these neurotoxins are and how they engender oxidative stress are the topics that we will discuss in this review. Conversely, how faithfully these neurotoxins model PD and how they should be used to achieve this goal will not be discussed. Readers interested in these latter aspects are encouraged to review other references (65, 66).

ROS-PRODUCING "PARKINSONIAN" NEUROTOXINS

Toxic models of PD are numerous, but thus far only a handful of such models have been thoroughly characterized with respect to their biochemical and molecular modes of action and neurodegenerative effects. Relatively well characterized models of PD include 6-hydroxydopamine (6-OHDA), paraquat, rotenone, and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (15). In principle, these toxins all share the same function, namely, the killing of SNpc dopaminergic neurons by a process in which oxidative stress is instrumental. Yet, as discussed below and depending on the neurotoxin, the molecular basis of the generated oxidative stress is quite different, and in broad terms 6-OHDA toxicity is dependent on its oxidation. The toxic action of paraquat is due to its reduction–oxidation cycling, whereas, at least in part, inhibition of the mitochondrial electron transport chain is responsible for the neurotoxicity of both rotenone and MPTP.

THE 6-OHDA MODEL: A PATRIARCH STILL IN THE RACE

6-OHDA was introduced as a catecholaminergic toxin >30 years ago (46) and, ever since, it has remained an extensively tested model both *in vitro* and *in vivo*. The effects of 6-OHDA on both the central and peripheral catecholaminergic pathways in rodents and in a variety of cultured cell types have been reviewed elsewhere, as well as the molecular basis for its specificity (45, 46, 65). 6-OHDA can be administered to rodents via a variety of different routes, but its proper utilization *in vivo* and *in vitro* relies on one's knowledge of a series of technical points that have been discussed in detail (45, 46, 65). Because of the emphasis of this special *Forum* of *Antioxidants & Redox Signaling* on oxidative stress in PD and experimental models of the disease and as 6-OHDA is a prototypical "oxidative-stress neurotoxin," we will focus the discussion on the 6-OHDA-induced neurotoxic mechanism. From the outset, it can be said that most experts agree on the concept that 6-OHDA destroys catecholaminergic structures by a combined effect of ROS and quinones (10). This popular view is based on the evidence that 6-OHDA, once dissolved in an alkaline solution, readily oxidizes in the presence of oxygen, yielding, in a stoichiometric fashion, hydrogen peroxide (H_2O_2) and *para*-quinone (37, 72) as depicted by the following reaction:

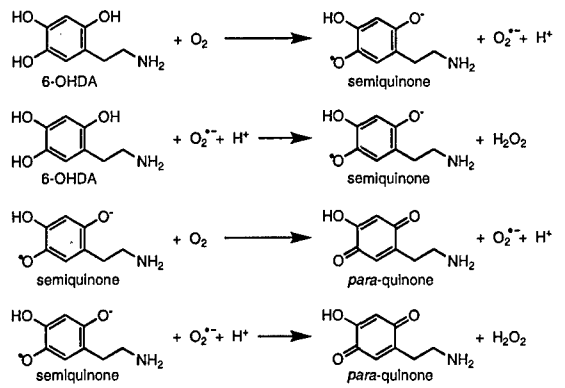


6-OHDA *para*-quinone

Although the chemical reaction that underlies 6-OHDA-induced neurotoxicity appears quite straightforward, it is in fact a remarkably complicated reaction that does not occur as a spontaneous oxidation by molecular oxygen. Still, molecular oxygen is mandatory for the reaction or, in anaerobic conditions, no conversion of 6-OHDA into quinones is detectable (30). If oxygen is necessary for the reaction, it is not, however, sufficient to drive 6-OHDA oxidation alone because desferrioxamine, a potent metal chelator, does inhibit the aerobic formation of 6-OHDA quinones to a dramatic extent (29–31, 80). This observation implies that 6-OHDA oxidation requires the presence of redox-capable transitional metals such as iron or copper to catalyze the transfer of electrons from 6-OHDA to molecular oxygen. It is now well accepted that even the presence of trace amounts of transitional metal contaminants, brought into the reaction mixture by the reagents and glassware, suffice to set this aerobic reaction in motion.

Aside from quinones, the oxidation of 6-OHDA also generates H_2O_2, as illustrated above. In biological systems, the production of H_2O_2 results from a two-electron reduction of oxygen. Thus, it can be surmised that during 6-OHDA oxidation a pair of electrons is transferred from 6-OHDA to molecular oxygen to produce H_2O_2. Yet it appears that the reaction of molecular oxygen with 6-OHDA is primarily a two-electron process only in the presence of excess oxygen, but it is a four-electron process in the presence of excess 6-OHDA (30). Accordingly, H_2O_2 is an end product of the reaction merely if 6-OHDA is limiting. Furthermore, even if the experimental conditions favor an overall exchange of a pair of electrons, the fact that oxygen has two unpaired electrons on its outermost orbital with a same spin quantum number makes it more likely that the reduction of oxygen proceeds by one electron at the time forming superoxide ($O_2^{\bullet -}$) and semiquinone radicals as the intermediary species. This interpretation is consistent with the demonstration that superoxide dismutase (SOD), by scavenging superoxide radicals, dramatically inhibits the oxidation of 6-OHDA (39). Subsequent studies have confirmed the production of superoxide radicals, and have moreover demonstrated that superoxide radicals generated by the first step of 6-OHDA oxidation are critical in propagating the oxidation of 6-OHDA (11, 30, 31, 80). As de-

tailed elsewhere (39), the progressive oxidation of 6-OHDA can be schematized as follows:



This shows that the oxidation of 2 moles of 6-OHDA leads to the formation of 2 moles of quinone and 2 moles of H_2O_2. In addition to the H_2O_2 and superoxide radicals, 6-OHDA oxidation is also associated with the production of hydroxyl radicals as demonstrated by using spin-trap 5,5-dimethyl-1-pyrroline-*N*-oxide (26) and methional as spin traps (11). In this system, hydroxyl radicals can arise from the Fenton reaction by which the breakdown of H_2O_2 is catalyzed by transitional metals such as iron.

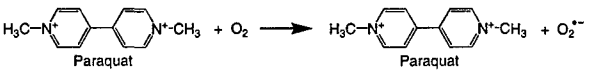
The above studies indicate that 6-OHDA oxidation generates not only *para*-quinone and H_2O_2, but also the superoxide and hydroxyl radicals. As stressed by many authors throughout this *Forum*, ROS such as H_2O_2, superoxide radical, and hydroxyl radical can either directly or indirectly inflict an array of cellular oxidations that can ultimately lead to cell death. Given this, the reader may encounter no difficulty envisioning how ROS generated by the oxidation of 6-OHDA could contribute to the neurotoxicity of this compound. On the other hand, how the quinone of 6-OHDA may exert deleterious effects may be less obvious. Early on in the characterization of the 6-OHDA mode of action, it was recognized that *para*-quinone formed though the oxidation of 6-OHDA undergoes covalent binding with sulfhydryl and other biological macromolecules with nucleophilic centers (32, 72). Accordingly, *para*-quinone is thus likely to react with glutathione and protein amino acid residues such as cysteine, tyrosine, and lysine. The deleterious consequences of the *para*-quinone of 6-OHDA may thus range from depletion of vital antioxidants such as glutathione, whose concentration is diminished in PD (64), to inactivation of critical enzymes such as catechol-*O*-methyltransferase (4) and tyrosine hydroxylase (49) and, more importantly, to an accumulation of potentially neurotoxic α-synuclein protofibrils, a proposed key event in PD pathogenesis (12).

Although the above-cited studies would argue that both the produced ROS and *para*-quinone are probably equally instrumental in the 6-OHDA neurotoxic processes, available evidence appears to favor the view that ROS are the dominant noxious mediators. For example, the addition of ascorbic acid to tissue slices, which is known to recycle *para*-quinone into 6-OHDA with a net formation of H_2O_2 (38, 80), prevents the appearance of colored quinones, but enhances neurotoxicity (38).

Finally, it should be emphasized that, like other monoamines, 6-OHDA can be metabolized by monoamine oxidase (MAO), a reaction that also generates ROS. This observation raises the possibility that the oxidative domination of 6-OHDA contributes to the neurotoxic process. Yet the finding that pretreatment with MAO inhibitors such as pargyline, rather than mitigating 6-OHDA toxicity, enhances it (45), argues against a MAO-dependent source of ROS as being contributive to the 6-OHDA neurotoxic process. It should also be stressed that, as long as the environmental conditions are favorable, oxidation of 6-OHDA can occur *in vivo* both intra- and extraneuronally. Consistent with this view is the demonstration that, in mesencephalic cultures, 6-OHDA toxicity is not restricted to dopaminergic neurons (55), and that several cell types devoid of transporters allowing 6-OHDA to be translocated inside the cell—such as C6 glioma, NIH-3T3, and CHO cells—can be damaged by this neurotoxin (3).

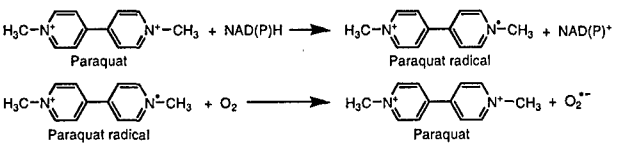
THE HERBICIDE PARAQUAT

The potent herbicide paraquat, whose chemical name is *N,N'*-dimethyl-4-4'-bipyridinium ion, is another prototypic toxin known to exert deleterious effects through oxidative mechanisms. Structurally, paraquat comprises two pyridine rings, *i.e.*, aromatic rings in which one carbon atom is replaced by a nitrogen atom, joined covalently by their number-4 carbon and with a methyl group attached to each nitrogen. The overall biochemical reaction governing the neurotoxic mechanism of paraquat was reported by Bus and collaborators roughly 30 years ago (6, 7). According to these authors, paraquat undergoes a single electron, reduction-oxidation cycling with subsequent formation of superoxide radicals:

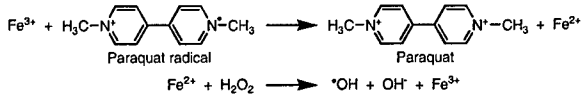


The first of the two steps of this biochemical reaction requires that paraquat go through a single-electron reduction to the blue-colored cation radical, paraquat$^{\bullet+}$ (28, 59). This initial step is not dependent on oxygen, as it can proceed under anaerobic conditions, but it does depend on the presence of diaphorase activity (28), *i.e.*, an enzyme that transfers an electron from a NAD(P)H molecule. Paraquat diaphorases are usually oxidoreductase enzymes containing flavin groups and using NADPH and, presumably, NADH as electron donors (9, 19, 22, 52, 77, 89). Relevant to the brain toxicity of paraquat, it should be noted that nitric oxide synthase (NOS) has been identified as one of the diaphorases capable of reacting with paraquat (19).

The second step of the paraquat toxic reaction is the reoxidation of this compound by oxygen that occurs through a transfer of a single electron from the paraquat radical to molecular oxygen, yielding oxidized paraquat (*i.e.*, the parent compound) and superoxide radicals. The actual reduction-oxidation cycling reaction of paraquat can thus be depicted as follow:



Of note, paraquat$^{\bullet+}$ is a powerful reducing radical capable not only of reacting with molecular oxygen to generate superoxide radicals as shown above, but also of reacting with transitional metals such as iron. Paraquat$^{\bullet+}$ can readily reduce iron(III) and most iron(III)chelates (9) into iron(II) or iron(II) chelates, which in turn could catalyze the formation of hydroxyl radicals via the Fenton reaction:



Presumably, whether paraquat$^{\bullet+}$ reacts with oxygen or iron depends on the concentration of oxygen. Accordingly, it can be speculated that in the brain the high oxygen content should favor the reaction of paraquat$^{\bullet+}$ with molecular oxygen over that with iron(III). Yet hydroxyl radicals can also be produced by an alternative mechanism in which paraquat$^{\bullet+}$ simply supplies the metal needed for the hydroxyl radical formation by reductively mobilizing iron from ferritin (83). Thus far, there is not an unequivocal demonstration that hydroxyl radicals are implicated in the deleterious effects caused by paraquat intoxication. Conversely, there are countless demonstrations that SOD overexpression and SOD mimetics confer resistance against paraquat (17, 18, 61), thus supporting the concept that superoxide radicals are pivotal in paraquat cytotoxicity.

Aside from oxidative stress, it has also been suggested that depletion of the intracellular stores of NAD(P)H, due to its increased oxidation during the reduction-oxidation cycling of paraquat stores, contributes to the overall cytotoxic process. Indeed, loss of NAD(P)H or change in the NAD(P)H over NAD(P)$^+$ ratio may have serious harmful consequences by impairing vital metabolic pathways such as fatty acid synthesis. Increased oxidation of glucose by the pentose phosphate shunt and inhibition of fatty acid synthesis in paraquat-intoxicated animals (79) are consistent with this view.

Most experimental studies of paraquat are related to its effects on the lungs, liver, and kidneys, probably because the toxicity induced by this herbicide in these organs is responsible for death after acute exposure. However, less known is the fact that significant damage to the brain is also seen in individuals who died from paraquat intoxication (33, 41) in spite of the fact that paraquat poorly crosses the blood–brain barrier (78). Thus far, the use of paraquat as a model of PD has been performed mainly in mice, perhaps because previous studies in rats have shown that oral administration of paraquat to these rodents was ineffective in damaging the brain (86) and that its intraventricular or intracerebral injection produces diffuse neurodegeneration (20, 53). Conversely, several authors have now reported reduced motor activity and dose-dependent losses of striatal dopaminergic nerve fibers and substantia nigra neuronal cell bodies in mice that received systemic injections of paraquat (5, 58, 82). Aside from killing dopaminergic neurons, paraquat induces α-synuclein upregulation and aggregation (56).

Quite surprising is the finding that dopaminergic but not other subpopulations of neurons, such as GABAergic cells in the substantia nigra and the striatum, appeared to be affected by paraquat injection (58). Neuronal subpopulations expressing high activity of NADPH-diaphorase in the brain are apparently not the dopaminergic neurons (63). Given the mechanism of action of paraquat, it is thus fascinating to note that dopaminergic neurons are specifically affected in this model.

THE MITOCHONDRIAL POISONS MPTP AND ROTENONE

Both MPTP and rotenone are well-known mitochondrial toxins used to recapitulate hallmarks of PD in laboratory animals (15). Although MPTP has been regarded for the past 20 years as the parkinsonian toxin *par excellence*, rotenone has recently received major attention for its capacity to produce previously unattainable PD features such as intraneuronal proteinaceous inclusions in rats (2). However, studies about the detailed mechanisms by which rotenone kills dopaminergic neurons are thus far few, which is in striking contrast with the large body of literature available on this topic for MPTP. Because it can be assumed that these two neurotoxins share many of the key molecular mechanisms responsible for their neurotoxicity, we will focus the rest of our discussion on MPTP and only refer to rotenone whenever published data permit.

A distinct feature of MPTP is that this neurotoxin is highly lipophilic and thus readily capable of crossing the blood–brain barrier after its systemic administration (57). Yet, once in the brain, MPTP as such is unable to provoke any neurotoxicity unless it is converted to 1-methyl-4-phenylpyridinium (MPP$^+$) (40, 57). The activation of the protoxin MPTP is a two-step process: first, MPTP is oxidatively deaminated by enzyme MAO-B to form the intermediate, unstable compound 1-methyl-4-phenyl-2,3-dihydropyridinium (MPDP$^+$); and second, MPDP$^+$ spontaneously oxidizes, yielding MPP$^+$ (Fig. 1). This process takes place not in dopaminergic neurons, which are devoid of MAO-B, but primarily in glial cells and secon-

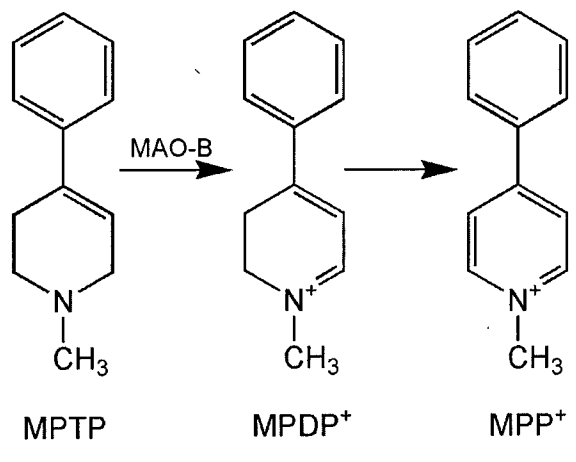


FIG. 1. Bioactivation of MPTP to MPP$^+$. The entire process of MPTP bioactivation takes place in serotonergic neurons and in astrocytes. The transformation of MPTP to MPDP$^+$ is catalyzed by MAO-B; the subsequent transformation of MPDP$^+$ to MPP$^+$ is spontaneous.

darily in serotonergic structures scarcely present in the vicinity of the nigrostriatal dopaminergic neurons. Once formed in these nondopaminergic cells, MPP^+ is released to the extracellular space, and through its binding to the plasma membrane dopamine transporter (44) it is translocated in dopaminergic neurons. Soon after its entry into dopaminergic neurons, MPP^+ participates in a variety of deleterious biochemical processes, among which many could generate oxidants. Although most of these oxidative reactions are taking place within the dopaminergic neuron itself, some meaningful reactive pathways originate from the surrounding glial cells. The current consensus in the field is that both the intrinsic and extrinsic oxidative stresses participate in the demise of nigrostriatal dopaminergic neurons in the MPTP model.

With respect to the intrinsic oxidative stress in the MPTP model, one of the main sources of the oxidant presumably emanates from the mitochondria. MPP^+, like rotenone, can accumulate within the mitochondria and bind to complex I of the electron transport chain (60). In doing so, MPP^+ interrupts the natural flow of electrons along this chain of cytochromes, which leads not only to an acute deficit in ATP formation, but also to an increased production of ROS, especially of superoxide (8, 35, 71). Because of the high amounts of Mn-SOD (SOD2) in the inner compartment of the mitochondria, it is likely that most, if not all, of the superoxide radicals produced by the blockade of complex I are immediately converted into H_2O_2. The latter, in contrast to the superoxide radical, could permeate through the mitochondrial membranes and thus can readily gain access to the cytosol. Accordingly, it is likely that mitochondrially generated superoxide may contribute to oxidative damage inside the mitochondria, whereas H_2O_2 may contribute to oxidative damage both inside and outside the mitochondria. These ROS may also engage in producing secondary and strong oxidants such as the hydroxyl radical, by reacting with an iron released from the destruction of mitochondrial aconitase (36), as well as with nitric oxide to generate peroxynitrite (43). Although there is little evidence that any of the reactive species cited above actually do inflict structural or functional mitochondrial damage in the MPTP model, the demonstration that transgenic mice with increased SOD2 activity are resistant to MPTP toxicity (47) argues that some type of MPP^+-mediated mitochondrial oxidative event has to be instrumental in the neurodegenerative process.

Presumably, ROS production can also occur in the MPTP model from the autooxidation of dopamine (54) resulting from an MPP^+-induced massive release of vesicular dopamine to the cytosol. Furthermore, the induction of cyclooxygenase-2 (COX-2) within the dopaminergic neurons after MPTP injection (42, 81) can also serve as a source of ROS. Indeed, via the peroxidase activity of COX-2, this enzyme can use catecholamines such as dopamine as an electron donor needed to catalyze the formation of dopamine-quinones. The latter may modify proteins by forming dopamine-cysteinyl adducts, which may have major consequences on the structure and function of modified proteins. In support of this scenario, we have found that, following MPTP injections to mice, contents of dopamine-cysteinyl in proteins increase markedly in a COX-2-dependent manner in affected brain regions (81).

The striking structural similarity between MPP^+ and paraquat (Fig. 2) has prompted several investigators to test the idea that MPP^+, like paraquat, could inflict oxidative stress via a reduction-oxidation cycling mechanism. Compared with paraquat, MPP^+ is an extremely stable species unlikely to undergo reduction–oxidation cycling (50). The reason paraquat is more reactive than MPP^+ relates to the double-positive charge on the paraquat, whereas MPP^+ has only one such charge (Fig. 2). For example, the one-electron reduction potential, which reflects the energy required to form the free radical, is -0.446 V for paraquat, well within the range of biological systems. In contrast, MPP^+ has a one-electron reduction potential of -1.18 V or greater, which is outside the range of known biological systems that might be involved in this reaction. Therefore, it seems unlikely that MPP^+ could participate in paraquat-like reduction–oxidation cycling unless an enzyme catalyzes it.

Although fierce discussions are still ongoing about which of these different sources of ROS, or combinations thereof, are implicated in MPTP neurotoxicity, there is compelling evidence that oxidative stress does play a critical role in the neurodegenerative process seen in this PD model. For instance, reduction of Cu/Zn-SOD (SOD1) activity by diethyl dithiocarbamate, which chelates copper and inhibits SOD1, or by genetic ablation of SOD1, potentiates MPTP-induced toxicity in mice (13, 90). The mirror opposite picture is found upon overexpressing human SOD1, in that transgenic mice with increased SOD1 activity are more resistant to MPTP (67). Although similar studies have not yet been done in rotenone, the toxicity of this other poison on dopaminergic cells appears also to implicate oxidative stress (74, 76).

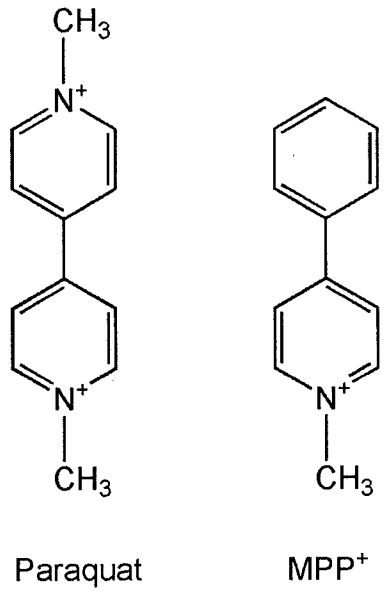


FIG. 2. Comparison of paraquat and MPP^+ chemical structures. Note the striking resemblance of the two compounds.

As already referred to above, ROS exert many or most of their toxic effects in the MPTP model in conjunction with other reactive species such as nitric oxide (1, 62, 69, 73) produced in the brain by both the neuronal and the inducible isoforms of the enzyme NOS (51, 68). A comprehensive review of the source and the role of nitric oxide in the MPTP model can be found in other references (63, 84).

Before discussing the extrinsic oxidative stress in the MPTP model, we should first emphasize the fact that the loss of dopaminergic neurons caused by both MPTP and rotenone is associated with a glial response (75, 85). Activation of microglia, which is quite pronounced in the MPTP and rotenone mouse models (14, 21, 48, 51, 75), reaches a maximum before the peak of dopaminergic neurodegeneration following the last MPTP injection (51). This observation has led to the idea that the MPTP- and rotenone-associated glial response may participate in the demise of dopaminergic neurons in these models. Studies showing that the blockade of microglial activation mitigates nigrostriatal damage caused by MPTP supports the notion that activated microglia participate in the neurodegenerative process (23, 87).

Activated microglial cells can produce a variety of noxious compounds, including ROS and RNS, proinflammatory cytokines, and prostaglandins. In many pathological settings, including MPTP injections, microglia activation involves the up-regulation of inducible NOS (21, 51) and the activation of NADPH oxidase (34). The former produces large amounts of nitric oxide in a calcium-independent manner, whereas the latter reduces oxygen to form superoxide radicals. Targeting inducible NOS by genetic interventions has shown that ablation of this enzyme, which reduces the production of nitric oxide, attenuates MPTP-induced neurotoxicity (21, 51). Similarly, mice defective in NADPH oxidase—and thus having reduced levels of extracellular superoxide—show less dopaminergic neuronal loss and protein oxidation than their wild-type littermates after MPTP injections (88). Further supporting the involvement of extracellular superoxide radicals in MPTP neurotoxicity is the finding that stereotaxic injection in the striatum of purified SOD1, which remains in the extracellular compartment, mitigates MPTP dopaminergic neurotoxicity on the infused side as compared with the noninfused side (88). Together, these findings indicate that the levels of extracellular nitric oxide and superoxide radicals are important components in the MPTP neurotoxic process.

CONCLUSIONS

This review summarized the molecular mechanisms underlying key neurotoxins used to model PD with a specific emphasis on oxidative stress. Whereas all four neurotoxins reviewed undoubtedly kill dopaminergic neurons, they all achieve this goal through different oxidative processes. By far the most complex of all appears to be that engendered by MPTP and, by analogy, probably by rotenone as well. If one is thus interested in the molecular biology behind dopaminergic neurotoxicity, it seems that MPTP, and by extension rotenone, may affect a greater variety of cellular pathways, perhaps making their study more appealing, but also more challenging. Nevertheless, whether the complexity of MPTP and rotenone oxidative processes more closely mimics the actual pathogenic cascade occurring in PD than the simpler oxidative processes engendered by 6-OHDA and paraquat is essentially unknown. Thus, if one is interested in testing new antioxidants for the treatment of PD, it may be necessary to preclinically ascertain the effectiveness of this putative neuroprotective intervention in more than one toxic model of PD.

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ABBREVIATIONS

COX-2, cyclooxygenase-2; H_2O_2, hydrogen peroxide; MAO, monoamine oxidase; $MPDP^+$, 1-methyl-4-phenyl-2,3-dihydropyridinium; MPP^+, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NOS, nitric oxide synthase; 6-OHDA, 6-hydroxydopamine; PD, Parkinson's disease; RNS, reactive nitrogen species; ROS, reactive oxygen species; SNpc, substantia nigra pars compacta; SOD, superoxide dismutase; SOD1, Cu/Zn-superoxide dismutase; SOD2, Mn-superoxide dismutase.

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Pathogenesis of nigral cell death in Parkinson's disease☆

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Abstract

Parkinson's disease (PD) is primarily a sporadic condition which results mainly from the death of dopaminergic neurons in the substantia nigra. Its etiology remains enigmatic while its pathogenesis begins to be understood as a multifactorial cascade of deleterious factors. As of yet, most insights into PD pathogenesis are derived from toxic models of PD and show that the earlier cellular perturbations arising in dopaminergic neurons include oxidative stress and energy crisis. These alterations, rather than killing neurons, trigger subsequent death-related molecular pathways including elements of apoptosis. The fate of dopaminergic neurons in PD may also be influenced by additional factors such as excitotoxicity, emanating from the increased glutamatergic input from the subthalamic nucleus to the substantia nigra, and the glial response that arises in the striatum and the substantia nigra. In rare instances, PD can be familial, and those genetic forms have also provided clues to the pathogenesis of nigrostriatal dopaminergic neuron death including abnormalities in the mechanisms of protein folding and degradation as well as mitochondrial function. Although more remains to be elucidated about the pathogenic cascade in PD, the compilation of all of the aforementioned alterations starts to shed light on why and how nigral dopaminergic neurons may degenerate in this prominent disease, that is PD.

Keywords: Parkinson's disease; MPTP; Neurodegeneration; Pathogenesis

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder after the Alzheimer's dementia. It is estimated that more than one million individuals in the United States of America alone are affected with this disabling disease and that more than 50,000 new cases arise each year [1]. PD is progressive with a mean age at onset of 55, and with an incidence that increases markedly with age [2]. Clinically, PD is characterized by the cardinal features of tremor at rest, slowness of voluntary movements, rigidity, and postural instability [1]. Like many other neurodegenerative diseases, PD presents itself mainly as a sporadic condition, meaning in absence of any genetic linkage, but in rare instances, PD can also arise as a simple Mendelian trait, linked to defects in a variety of genes [3]. Although, clinically and pathologically, sporadic and familial PD may differ on several significant aspects, they all share the same biochemical brain abnormality, namely a dramatic depletion in brain dopamine [2].

The reason why PD patients exhibit low levels of brain dopamine stems from the degeneration of the nigrostriatal dopaminergic pathway, which is made of dopaminergic neurons whose cell bodies are located in the substantia nigra pars compact and whose projecting axons and nerve terminals are found in the striatum [2]. Yet, it is important to emphasize the fact that the neuropathology of PD is far from being restricted to the nigrostriatal pathway, and histological abnormalities can be found in many other dopaminergic and even non-dopaminergic cell groups [2]. The second most prominent neuropathological feature of PD is the presence of intraneuronal inclusions called Lewy bodies (LBs) in the few remaining nigral dopaminergic neurons [2]. LBs are spherical eosinophilic cytoplasmic aggregates composed of a variety of proteins, such as α-synuclein, parkin, ubiquitin and neurofilaments, and they can be found in every affected brain region [2].

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Over the past few decades a large core of data originating from clinical studies, autopsy materials, and in vitro and in vivo experimental models of PD has been accumulated, which led us to begin to have some level of understanding of the pathogenesis of sporadic PD [2]. Available data would argue that the mechanism of neuronal death in PD starts with an otherwise healthy dopaminergic neuron being hit by an etiological factor, such as mutant α-synuclein. Subsequent to this initial event, it is proposed that a cascade of deleterious factors is set in motion within that neuron made not of one, but rather of multiple factors such as free radicals, mitochondrial dysfunction, excitotoxicity, neuroinflammation, and apoptosis to cite only some of the most salient. Still based on this proposed scenario, all of these noxious factors will interact with each other to ultimately provoke the demise of the injured neuron.

Despite unquestionable major advances made in the molecular and cellular biology of PD and other neurodegenerative diseases which brought us closer than ever to being capable of unraveling the pathogenesis of PD, several critical questions remain unanswered. In this paper, three pending questions pertinent to the mechanisms of neuronal death in PD are discussed and form the body of this review. To be discussed first will be the question of what do we know about the nature of the pivotal factors and the sequence in which they act within the proposed pathogenic cascade that leads to neuronal death in PD. Second is the question to know whether the overall neurodegenerative process in PD is truly a cell autonomous process will be briefly addressed. Finally, one cannot avoid discussing the contribution of rare, inherited forms of PD to our current understanding of the pathogenesis of sporadic of PD.

2. Nature and sequence of action of pathogenic factors in PD

The current model of pathogenesis that most investigators in the field utilize has been outlined above. To confirm the actual role of these different presumed factors and the sequence by which they, respectively, intervene in this multifactorial cascade has been primarily, if not exclusively, studied in toxic experimental models of PD, which are numerous. Findings from these models and especially from that produced by the parkinsonian neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) indicate that the initial cellular perturbations include inhibition of mitochondrial respiration. Indeed, soon after the systemic administration of MPTP to mice, its active metabolite, 1-methyl-4-phenylpyridinium ion (MPP^+), does concentrate in the mitochondrial matrix, where it binds to complex I of the electron transport chain [4]. MPP^+ binding interrupts the flow of electrons along the mitochondrial electron transport chain, thereby leading to an increased production of reactive oxygen species (ROS), especially of superoxide radicals [4]. Previous work has clearly demonstrated that the magnitude of MPP^+-related ROS production within the mitochondria is a dose-dependent phenomenon [5]. The pathogenic significance of such a local excess of ROS production is supported by the demonstration that mitochondrial aconitase activity is reduced in ventral midbrain of MPTP-treated mice [6]. MPP^+-related loss of electron flow is also associated with a drop in ATP production [4], which in vivo is found only in susceptible areas of the brain such as ventral midbrain and striatum [7]. Remarkably, this work shows that ATP deficit develops very rapidly after MPTP injection and lasts only for a few hours, as by one day post-MPTP tissue content in ATP seem to return to normal values [7].

In addition to provoking mitochondrial oxidative stress and energy crisis, MPP^+ also interacts with synaptic vesicles through its binding to vesicular monoamine transporter-2 [8]. In so doing, MPP^+ translocates into synaptic vesicles where it stimulates the extrusion of synaptic dopamine [9,10], reminiscent of the effect of methamphetamine. The resulting excess of cytosolic dopamine can readily undergo autooxidation, thus generating a huge burst of ROS, subjecting nigral neurons to an oxidative stress [11]. Alternatively, oxidation of cytosolic dopamine can also be catalyzed by enzymes such as cyclooxygenase-2 [12], which is upregulated in the remaining nigral dopaminergic neurons in both MPTP-treated mice and in human post-mortem samples [13]. Supporting this proposed event is our demonstration that cyclooxygenase-2 promotes dopamine–quinone formation following MPTP injection, and the production of protein-bound 5-cysteinyl-dopamine adducts in the brain of MPTP-injected mice [13]. In addition, excess of cytosolic dopamine can stimulate the formation of neuromelanin [14], a dark intraneuronal pigment implicated in the greater susceptibility of nigral neurons to PD neurodegeneration [15]. Although it remains uncertain how neuromelanin does actually contribute to the demise of dopaminergic neurons, it has been hypothesized that this pigment can do so by a *macromolecule crowding effect* or by playing the role of *intraneuronal toxic reservoir* by binding different transitional metals, such as iron, and various toxicants, such as MPP^+ [16].

All of the studies discussed above point toward the superoxide radical being pivotal in MPTP neurotoxicity. However, superoxide radical is known to not be highly reactive, and thus it is unlikely that it may be directly responsible for the damage inflicted by MPTP. Instead, it is much more likely that superoxide neurotoxicity results from superoxide reacting with other reactive molecules to generate what are called *secondary reactive species* of much greater tissue damaging potential, such as peroxynitrite. Consistent with this view are the demonstrations that the production of peroxynitrite, evidenced by quantifying tissue content of protein-bound 3-nitrotyrosine, is increased after MPTP injection [17] and that peroxynitrite is likely implicated in the nitrative post-translational modifications

of pathogenically meaningful proteins such as α-synuclein and parkin [18,19].

Collectively, the aforementioned findings indicate that early pathogenic events following MPTP administration include mitochondrial and cytosolic oxidative stress and ATP deficit. Yet, when one compares the time course of these cellular perturbations with the actual phase of neuronal degeneration found after MPTP injections, it clearly appears that oxidative stress and energy crisis precede the peak of dopaminergic neuronal death in the substantia nigra of mice which is situated around 24–48 h after the last injection of MPTP [20]. This finding suggests that these early events may kill some dopaminergic neurons, but that most of the neurons injured by this parkinsonian toxin fail to succumb to this early attack. Instead, it is believed that rather than killing a large number of neurons, early oxidative stress and energy crisis activate cell death-related molecular pathways which are the real executioner of the injured neurons. Among these are c-Jun *N*-kinases [21], cyclin-dependent kinases [22], and various components of the apoptotic machinery [23]. To illustrate the critical role of these molecular pathways in the MPTP-induced neurodegenerative process, two studies that pertain to apoptosis will be discussed here. First is the work done on the pro-cell death protein Bax, demonstrating that not only is Bax highly expressed in nigral dopaminergic neurons, but that ablation of Bax renders mice more resistant to the dopaminergic neurotoxicity of MPTP [24]. The second study deals with apoptotic protease activating factor 1 (APAF-1), one of the critical components of the apoptosome complex [25]. In the latter work, the authors have unilaterally delivered a viral vector expressing a dominant negative mutant of APAF-1 by stereotaxic injection in the substantia nigra [25]. Then, they have subjected these mice to a systemic administration of MPTP and found that the blockade of APAF-1 did mitigate the death of dopaminergic neurons [25]. These two studies clearly demonstrate the importance of downstream molecular pathways such as apoptosis in the death of nigral dopaminergic neurons and are consistent with the sequential pathogenic model proposed above.

3. Is the neurodegenerative process in PD cell autonomous?

This question is of critical importance both for pathogenic and therapeutic reasons. Indeed, it is quite important to determine whether the demise of nigral dopaminergic neurons strictly results from the cellular perturbations that arise within these neurons due to the disease's etiology, or from a complex interaction between what are called intrinsic and extrinsic perturbations. The answer to this question is complicated and far from straightforward. For instance, if one reviews the body of literature on cultured neurons exposed to MPP^+ or overexpressing PD-causing proteins such as α-synuclein [2], there is no doubt that these catecholaminergic neurons die in absence of any other intervening exogenous factors such as other cell types. However, when one looks at more complex systems, such as post-mortem tissues from PD patients or in vivo experimental models of PD, there is mounting evidence that indicates that the surroundings of the nigral dopaminergic neurons appear to play a critical role in influencing the fate of these dopaminergic cells. Among the potential culprits is the increased glutamatergic input to the nigra and which originates from the hyperactive subthalamic nucleus [26,27]. Moreover, the glial response that is found in both striatum and nigra of PD patients and MPTP-mice is also likely to exert deleterious effects on the remaining dopaminergic neurons [28]. This view has led many investigators, including those in my laboratory, to aggressively examine the potential role of neuroinflammation in the pathogenesis of PD. This important topic, however, will not be reviewed here as it is discussed in-depth in the accompanied paper written by Dr E. Hirsch. Based on these data and those presented by Dr Hirsch in this special issue, it is our opinion that several factors, exterior to dopaminergic neurons, contribute to creating a hostile environment, which increases the stress on already compromised dopaminergic neurons present in the vicinity. These factors, while likely not capable of initiating the disease, are nevertheless likely to amplify the neurodegenerative process and stimulate the progression of a chronic disease such as PD. If this view is correct, one then must take into account those exogenous factors if one wishes to completely and accurately comprehend the pathogenic cascade underlying the neurodegenerative process of PD and to develop effective neuroprotective therapies for this illness.

4. Insights from the rare inherited forms of PD

Until recently, all of the hypotheses regarding the cause and the mechanisms of PD neurodegeneration came from investigations performed in autopsy material from sporadic PD cases or in neurotoxic models [2]. However, less than a decade ago this situation changed with the identification of a mutation in α-synuclein associated with PD in an Italian kindred [29]. Since then, four additional PD-causing genes have been identified, and a linkage has been reported for at least five more. Although rare, these inherited cases have opened new directions of research which have already led to the integration within the proposed pathogenic cascade of new molecular components. In particular, familial cases of PD have brought to our attention the potential importance of protein aggregation and abnormalities in protein turnover in the overall process provoking neurodegeneration in PD. The contribution to our understanding of the pathogenesis of sporadic PD from the different PD-causing mutations has been discussed elsewhere [3], and thus readers

interested in this question are urged to consult this paper. Since the publication of this latter review, several important new findings have been published and, of these, three are worth discussing briefly.

As mentioned above, protein degradation has emerged as a potentially important theme in PD pathogenesis, especially in the context of alterations of the proteasome/ubiquitin pathway. Yet, protein degradation does not solely rely on the proteasome/ubiquitin pathway, but also on autophagy. With respect to autophagy, it has been reported that both wild-type and mutant α-synuclein can be degraded by lysosomal enzymes and that both bind to the autophagy chaperone [30]. Remarkably, however, mutant α-synuclein binds with much greater avidity to the autophagy chaperone than its wild-type counterpart [30]. Furthermore, contrary to wild-type α-synuclein which after binding to the chaperone is rapidly taken up by autophagic vacuoles for lysosomal degradation, mutant α-synuclein remains tightly attached to the chaperone and is never taken up or degraded [30]. These striking observations indicate that mutant α-synuclein fails to be properly degraded by the chaperone-mediated autophagy (CMA). It can, thus, be speculated that part of the neurotoxic mechanism of mutant α-synuclein may be related to the blockade of CMA and the consequent accumulation of unwanted proteins that are no longer eliminated by CMA.

The two other studies to be discussed here pertain to observations made on DJ1 and PINK1, the products of genes which upon mutation are linked to familial forms of PD, whose sub-cellular locations are mitochondrial [31,32]. In both cases, it was shown that either the abrogation of the mitochondrial localization or the loss of activity of these proteins renders cells subjected to mitochondrial poisons or proteasome inhibitor more prompt to degeneration [31,32]. Although the exact functions of DJ1 and PINK1 remain to be elucidated, these results already indicate that wild-type DJ1 and PINK1 assume some type of mitochondrial functions which confer resistance of the whole cell to a variety of stressors. Worth noting is the fact that, at this point there is no evidence to indicate that the putative mitochondrial action of DJ1 or PINK1 is linked to the electron transport chain machinery.

5. Conclusion

In this short review, an attempt has been made to stress the fact that the current consensus regarding the pathogenesis of sporadic PD is based primarily on information gathered from neurotoxic models of the disease. Based on these data, it appears that nigral dopaminergic neuron degeneration does not result from the action of a single deleterious factor, but rather from the convergence of multiple pathogenic factors. Many of these noxious factors emanate from within the dopaminergic neurons, whereas several others originate from outside the dopaminergic neurons such as glutamatergic input and glial cells.

We also know by now that while uncommon, there is much to learn from these rare familial cases of PD linked to gene mutations. In a matter of a few years, thanks to several elegant investigations performed in genetic cases of PD, we have become aware of the importance of excess protein aggregation with respect to mechanisms of neuronal death, perturbations in protein degradation systems such as proteasome and autophagy, and accumulation of unwanted proteins. These genetic cases have also shed light on new mitochondrial mechanisms other than those related to the electron transport chain which may have great pathogenic significance.

Despite enormous advances, it is fair to conclude by saying that much remains to be done to completely unravel the pathogenesis of PD. Among specific aspects that may deserve particular attention are the identification of where within the proposed pathogenic cascade do mutation-related deleterious mechanisms intersect with those mediated by the parkinsonian toxins. Why nigral neurons are more vulnerable than other dopaminergic neurons to the PD neurodegenerative process is also paramount to a comprehensive understanding of the neurobiology of this prominent neurodegenerative disease.

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Complex I deficiency primes Bax-dependent neuronal apoptosis through mitochondrial oxidative damage

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Dysfunction of mitochondrial complex I is a feature of human neurodegenerative diseases such as Leber hereditary optic neuropathy and Parkinson's disease. This mitochondrial defect is associated with a recruitment of the mitochondrial-dependent apoptotic pathway *in vivo*. However, in isolated brain mitochondria, complex I dysfunction caused by either pharmacological or genetic means fails to directly activate this cell death pathway. Instead, deficits of complex I stimulate intramitochondrial oxidative stress, which, in turn, increase the releasable soluble pool of cytochrome *c* within the mitochondrial intermembrane space. Upon mitochondrial permeabilization by the cell death agonist Bax, more cytochrome *c* is released to the cytosol from brain mitochondria with impaired complex I activity. Given these results, we propose a model in which defects of complex I lower the threshold for activation of mitochondrial-dependent apoptosis by Bax, thereby rendering compromised neurons more prone to degenerate. This molecular scenario may have far-reaching implications for the development of effective neuroprotective therapies for these incurable illnesses.

mitochondria | neurodegeneration | Parkinson's disease

Reduced activity in mitochondrial complex I (NADH/ubiquinone oxidoreductase) is associated with a wide spectrum of neurodegenerative diseases (1). Low complex I activity due to mitochondrial DNA point mutations is found in many cases of Leber hereditary optic neuropathy, which is characterized by a massive retinal ganglion cell degeneration resulting in a rapid loss of central vision (2). Reduced complex I activity has also been reported in both autopsy brain tissues and platelets of patients affected with sporadic Parkinson's disease (PD) (3, 4). The pathogenic role of this mitochondrial dysfunction is supported by demonstrations that natural and synthetic complex I antagonists provoke neuronal death in animals (5–7). The molecular basis of neuronal death mediated by defective complex I activity is just beginning to be deciphered, in part by the utilization of the mitochondrial poisons 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and rotenone. For instance, it is now established that inhibition of complex I in rodents leads to degeneration of dopaminergic neurons of the substantia nigra pars compacta, as seen in PD (5), through activation of apoptotic molecular pathways (8–10). Moreover, it is believed that complex I dysfunction and the subsequent impairment of mitochondrial respiration provoke the activation of the mitochondrial-dependent apoptotic machinery by directly triggering the release of the apoptogenic molecule cytochrome *c* from the defective mitochondria (11–14).

Here we show that, contrary to the proposed direct effect of complex I deficit on cytochrome *c* release and consequent cell death, complex I defects do not autonomously recruit the apoptotic machinery. Instead, we show that complex I deficiency sensitizes neurons to mitochondrial-dependent apoptosis in response to the cell death agonist Bax through mitochondrial oxidative damage, by increasing the releasable soluble pool of cytochrome *c* within the mitochondrial intermembrane space. This molecular scenario sheds light into the mechanisms of cell death in chronic diseases linked to complex I deficiency and may have far-reaching implications for the development of new neuroprotective therapies for these incurable illnesses.

Materials and Methods

Animals and Treatment. Eight-week-old wild-type or Bax-deficient male mice received one i.p. injection of MPTP-HCl per day (30 mg/kg per day of free base; Sigma-Aldrich) for 5 consecutive days and were killed at 0, 2, 4, 7, 21, and 42 days after the last injection; control mice received saline injections only (n = 3–10 mice per time point, treatment, and genotype).

Subcellular Fractionation. Protein extraction of mitochondrial and cytosolic fractions was performed on fresh ventral midbrain tissue from saline- and MPTP-injected mice, as described (15).

Antibodies. The following primary antibodies were used for Western blot analysis: mouse monoclonal anti-cytochrome *c* (PharMingen); mouse monoclonal anti-cytochrome *c* oxidase-IV (Molecular Probes); rabbit polyclonal anti-cleaved caspase-3 (CM1; Idun Pharmaceuticals, La Jolla, CA); rabbit polyclonal anti-cleaved caspase-9 (Asp-353; Cell Signaling Technology, Beverly, MA); mouse monoclonal anti-β-actin (clone AC15; Sigma); mouse monoclonal anti-Bax (B-9; Santa Cruz Biotechnology); rabbit polyclonal anti-sulfite oxidase (gift from J. L. Johnson, Duke University Medical Center, Durham, NC); and goat polyclonal anti-HSP60 (Santa Cruz Biotechnology).

Immunofluorescence. For double immunofluorescence and confocal microscopy, a mouse monoclonal anti-cytochrome *c* (catalog no. 556432; PharMingen) and a rabbit polyclonal anti-adenine nucleotide translocase-1 (ANT-1; Oncogene, Boston) were used. Distribution of the fluorescent signal for both ANT-1 and cytochrome *c* stainings was analyzed by using the IMAGEJ 1.28U software (National Institutes of Health), similarly as de-

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Abbreviations: PD, Parkinson's disease; MPTP, 1-methyl-4-pheny-1,2,3,6-tetrahydropyridine; ROS, reactive oxygen species; ANT-1, adenine nucleotide translocase-1; MPP^+, 1-methyl-4-phenylpyridinum ion; TMPD, *N,N,N',N'*-tetramethyl-1,4-benzenediamine.

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extraction, polarography, H_2O_2 production and cytochrome *c* release studies were performed as described above.

Ascorbate/*N,N,N′,N′*-Tetramethyl-1,4-Benzenediamine (TMPD) Assay. These experiments were performed as described (20). Briefly, 1 mg/ml mitochondria was incubated in sucrose buffer (0.2 M sucrose/10 mM Trisphosphate-4-morpholinepropanesulfonic acid, pH 7.4/1 mM Pi/5 mM glutamate/2.5 mM malate/10 μM EGTA-phosphate Tris, pH 7.4) and treated as indicated in Fig. 4. After the indicated time, 400 pmol carbonyl cyanide 3-chlorophenylhydrazone (CCCP) and 1 nmol antimycin A per mg of protein^{-1} were added, and the reaction was transferred to a Clark-type oxygen electrode chamber. Final volume was 1 ml at 25°C. After 2 min, 6 mM ascorbate was added, followed by 300 μM TMPD 3 min thereafter. The ascorbate-driven oxygen consumption rate over the total TMPD-driven rate is plotted as a percentage of the ratio in the untreated mitochondria.

MPP$^+$–Cardiolipin Interaction Assay. Different amounts of [^{3}H]-MPP$^+$ were incubated in a cardiolipin-coated ELISA plate (Alpha Diagnostics, San Antonio, TX) for 15 min. Residual radioactivity was measured after washing out radioactive MPP$^+$.

Lipid Extraction and HPLC. Lipids from isolated brain mitochondria were extracted as described (21). Lipid extraction from midbrain, striatal, and cerebellar brain mitochondria was performed by pooling the above-mentioned anatomical regions from five different saline- or MPTP-intoxicated mice. The HPLC measurements were carried out by using an adaptation of a previously described method (21).

Statistical Analysis. All values are expressed as the mean ± SEM. Differences among means were analyzed by using one- or two-way ANOVA with time, treatment, or genotype as the independent factors. When ANOVA showed significant differences, pair-wise comparisons between means were tested by Student–Newman–Keuls post hoc testing. In all analyses, the null hypothesis was rejected at the 0.05 level.

Results

Complex I Inhibition Relies on Bax to Engage Mitochondrial-Dependent Apoptosis. Bax, a proapoptotic member of the Bcl-2 family, plays a critical role in the demise of dopaminergic neurons provoked by complex I inhibition (8). In most circumstances, including the pharmacological blockade of complex I (22), Bax-mediated cell death is accompanied with a mitochondrial release of cytochrome *c* and activation of caspase-9 and -3. We confirmed that mitochondrial release of cytochrome *c* does occur in MPTP-intoxicated mice (Fig. 1). This molecular event is time-dependent and coincides with the induction (8) and relocation of Bax from the cytosol to the mitochondria (Fig. 1). We also show that Bax mitochondrial translocation and cytochrome *c* release parallel the activation of downstream caspases (Fig. 1) and the previously reported time course of neuronal apoptosis caused by MPTP (8). Supporting the pivotal role of Bax in this molecular cascade is the demonstration that the release of cytochrome *c* and the activation of caspases are absent in Bax-deficient mutant mice treated with MPTP (Fig. 1 *f* and *g*); these mice were previously found resistant to MPTP-induced neurodegeneration (8). However, abrogation of Bax in mutant mice did not impair the potency of MPTP to inhibit complex I (Fig. 6, which is published as supporting information on the PNAS web site). Our results thus indicate that complex I deficiency operates together with Bax to engage the mitochondrial-dependent apoptotic pathway *in vivo*.

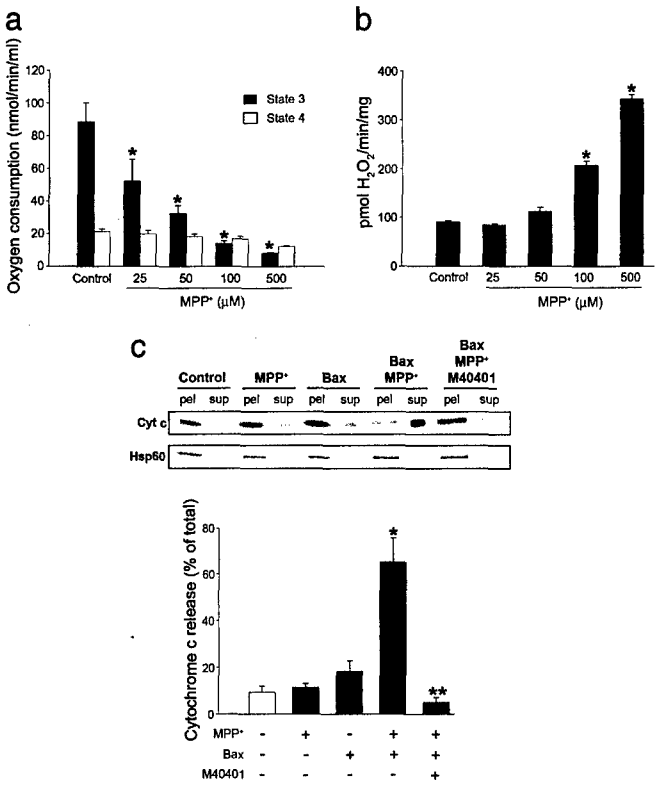

Fig. 2. Complex I inhibition stimulates ROS production and promotes Bax-dependent cytochrome *c* release in isolated brain mitochondria. (*a*) MPP$^+$ induces a dose-dependent inhibition of complex I-driven mitochondrial respiration, as assessed by monitoring oxygen consumption after addition of ADP, which in normal mitochondria induces a transient mitochondrial depolarization with a subsequent burst of oxygen consumption (state 3 respiration) until the added ADP is converted to ATP (state 4). (*b*) Complex I inhibition by MPP$^+$ induces dose-dependent ROS production in brain mitochondria, as assessed by measuring H_2O_2 using the fluorescent dye Amplex Red. (*c*) Complex I inhibition with 100 μM MPP$^+$ or incubation with ≈100 nM recombinant Bax, alone did not trigger significant release of cytochrome c from isolated brain mitochondria. However, combining complex I inhibition with recombinant Bax resulted in a marked release (>60%) of cytochrome *c*. This effect was abolished by 50 μM of the superoxide dismutase mimetic M40401. Matrix mitochondrial protein HSP60 was not mobilized by any of the tested conditions. *, $P < 0.05$, compared with untreated mitochondria; **, $P < 0.05$, compared with mitochondria treated with MPP$^+$ and recombinant Bax.

Inhibition of Complex I Potentiates Bax-Induced Cytochrome *c* Release. We next ascertained in isolated brain mitochondria the respective roles of complex I deficiency and Bax activation in the recruitment of the mitochondrial-dependent neuron death program, as well as the molecular basis for their interaction. First, we incubated purified brain mitochondria with different concentrations of MPTP's active metabolite, MPP$^+$, or rotenone. These experiments confirmed that both MPP$^+$ and rotenone caused, in a dose-dependent manner: (*i*) a reduction of ADP-stimulated oxygen consumption (state 3 respiration) supported by the NADH-linked substrates glutamate/malate (Fig. 2*a* and Fig. 7, which is published as supporting information on the PNAS web site); and (*ii*) an increased production of reactive oxygen species (ROS) (Figs. 2*b* and 7), likely generated by a higher rate of molecular oxygen reduction into superoxide radical in response to the hampered terminal step of electron transfer from the highest potential iron–sulfur cluster of complex I to ubiquinone (23). Contrary to the effect of complex I deficiency on cytochrome *c* release in intact cells *in vivo*, we found no evidence that MPP$^+$- or rotenone-induced complex I inhibition elicited a release of cytochrome *c* from purified brain mitochondria, even

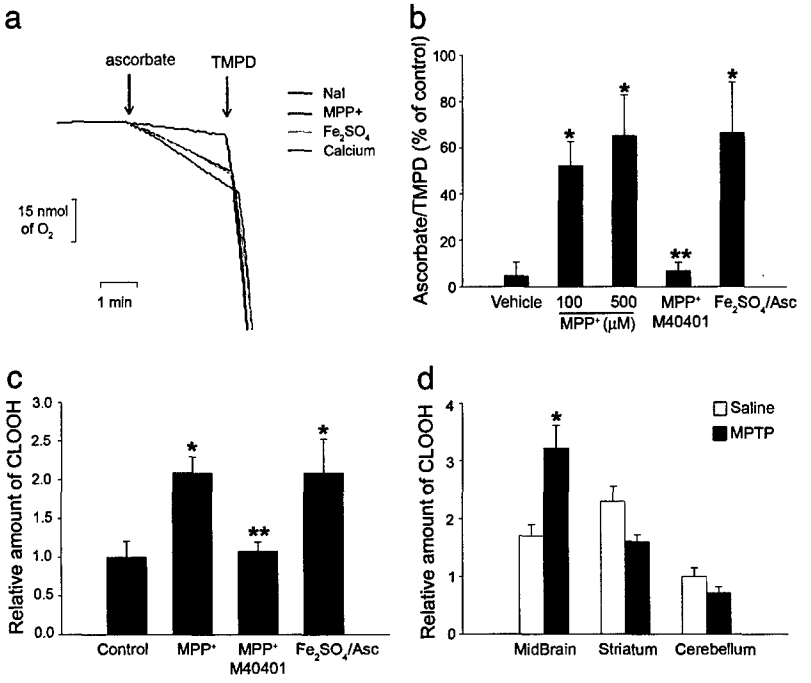

Fig. 4. Complex I inhibition increases the soluble pool of cytochrome *c* in the mitochondrial intermembrane space by oxidizing cardiolipin. (*a* and *b*) Complex I inhibition by MPP^+ induced a dose-dependent increase of mitochondrial ascorbate/TMPD-driven respiration ratio, consistent with an increased intermembrane soluble pool of cytochrome *c*. This effect was prevented by 50 μM M40401 and could be reproduced by the ROS-generating compound, Fe_2SO_4 (60 μM)/ascorbate (500 μM). Sodium iodide (NaI) was used as vehicle, because MPP^+ was used in the form of MPP^+-I. Ca^{2+}-mediated mitochondrial swelling, which increases the soluble pool of cytochrome *c* (20), was used as a positive control. (*c*) Complex I inhibition by MPP^+ induced oxidation of inner mitochondrial membrane cardiolipin in isolated brain mitochondria, as assessed by determining cardiolipin hydroperoxide (CLOOH) content by HPLC. Oxidation of cardiolipin was also produced by Fe_2SO_4/ascorbate and was attenuated by M40401. (*d*) Oxidized cardiolipin was detected in ventral midbrain samples from MPTP-intoxicated mice but not in regions devoided of MPTP-induced cell loss, such as striatum and cerebellum. *, $P < 0.05$, compared with controls; **, $P < 0.05$, compared with MPP^+-treated mitochondria.

mitochondrial ascorbate/TMPD-driven respiration ratio (Fig. 4 *a* and *b*), consistent with an increased intermembrane space soluble pool of cytochrome *c*. This effect was also prevented by M40401 (Fig. 4*b*) and could be reproduced by Fe_2SO_4/ascorbate (Fig. 4 *a* and *b*), indicating its dependency on ROS production.

ROS Generated by Complex I Inhibition Oxidize the Inner Mitochondrial Lipid Cardiolipin both *in Vitro* and *in Vivo*. It has been shown in isolated rat liver mitochondria that cytochrome *c* can be freed from the inner mitochondrial membrane by oxidative modifications of cardiolipin (21). Therefore, we tested whether cardiolipin peroxidation occurred after complex I blockade. After having excluded the possibility that MPP^+ displaces cytochrome *c* from the negatively charged cardiolipin by simple electrostatic interaction (see *Materials and Methods*), we assessed by HPLC the contents of oxidized cardiolipin in isolated brain mitochondria. Complex I blockade by MPP^+ resulted in a marked increase of oxidized cardiolipin that was attenuated by M40401, indicating its dependency on ROS production (Fig. 4*c*). Furthermore, incubation of isolated brain mitochondria with the ROS-generating system Fe_2SO_4/ascorbate also increased cardiolipin oxidation (Fig. 4*c*). Supporting a role in neurodegeneration induced *in vivo* by complex I inhibition, cardiolipin oxidation was also detected in mitochondria isolated from ventral midbrain of mice intoxicated with MPTP (Fig. 4*d*) in a time-dependent manner that preceded activation of Bax and apoptotic neuron death in this model of complex I deficiency (8).

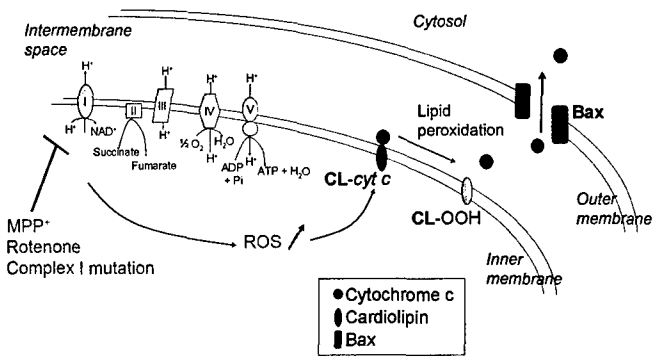

Fig. 5. Proposed pathogenic scenario induced by complex I deficiency. Pharmacological or genetic inhibition of complex I disrupts mitochondrial respiration and stimulates the mitochondrial production of ROS. As a consequence, an array of molecules is likely oxidatively modified in response to complex I defect, including the inner mitochondrial membrane lipid cardiolipin. Cardiolipin peroxidation, in turn, affects the binding of cytochrome *c* to the mitochondrial inner membrane, leading to an increased soluble pool of cytochrome *c* in the intermembrane space. Consequently, upon permeabilization of the outer mitochondrial membrane by activated Bax, a larger amount of mitochondrial cytochrome *c* can be released, making it more likely for a compromised neuron to undergo apoptosis (see *Discussion* for more details).

Discussion

The mitochondrial-dependent apoptotic pathway has been shown to be instrumental in the neuronal degeneration associated with disruption of mitochondrial respiration caused by complex I deficiency, as demonstrated by targeting molecules of this pathway such as Bax, caspase-9, or Apaf-1 (8, 22, 28). Our study, while confirming that complex I defects lead to a recruitment of the mitochondrial-dependent apoptotic pathway *in vivo*, sheds light onto the molecular mechanisms linking these two events. For instance, after MPTP administration, there is indeed a time-dependent and region-specific mitochondrial release of cytochrome *c* that occurs in association with activation of both caspase-9 and -3. All of these molecular alterations appear to be regulated by the death agonist Bax, because they coincide with Bax up-regulation and translocation to the mitochondria and are prevented by genetic ablation of Bax. Although Bax induction was previously shown to rely on p53 activation after complex I inhibition (29), the mechanism driving Bax mitochondrial translocation after complex I inhibition remains to be determined (10). Both Bid and Bak are known for cooperating with Bax to initiate mitochondrial-dependent apoptosis in response to the ligation of cell-surface death receptors. However, in contrast to the pivotal role of Bax, both Bid and Bak have been shown to be dispensable in complex I deficiency-mediated neuronal death (22, 30).

Our study also clarifies the process by which complex I defects contribute to the actual recruitment of the mitochondrial-

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Review

The MPTP model of Parkinson's disease

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Abstract

The biochemical and cellular changes that occur following administration of 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) are remarkably similar to that seen in idiopathic Parkinson's disease (PD). In this review, we detail the molecular activities of this compound from peripheral intoxication through its various biotransformations. In addition, we detail the interplay that occurs between the different cellular compartments (neurons and glia) that eventually consort to kill substantia nigra pars compacta (SNpc) neurons.

Keywords: Parkinson's disease; Glial cells; Substantia nigra; MPTP

Contents

1. Introduction

Parkinson's disease (PD) was first described in a paper entitled "An Essay on the Shaking Palsy" in 1817 by Dr. James Parkinson in Ref. [109]. PD is a progressive neurological disorder that strikes 1–2% of the "over 50" population [21]. Current estimates from the American Parkinson's Disease Foundation put the number of American citizens suffering from this disease at greater than 1.5 million persons. At this time, PD is the third most prevalent neurodegenerative disorder, following Alzheimer's disease and dementia with Lewy body disease. Since the disease incidence increases with age, it is likely that the number of people suffering from PD will rise as improved healthcare lengthens the average life span.

The main anatomical feature of PD is the decrease in number of neuromelanin-containing neurons located in the midbrain substantia nigra pars compacta (SNpc). These dopaminergic neurons project to the striatum as well as a number of other subcortical regions [161]. PD symptoms first manifest when approximately 60% of the SNpc neurons have already died [39] and 70% of dopamine responsiveness disappears [83]. Because the progression of cell loss is thought to occur over a somewhat protracted period of time in a defined spatiotemporal manner [18,104],

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the onset of Parkinson's disease symptoms is often insidious.

At this time, it is unclear as to how much of the disease results from a purely environmental factor, a strict genetic causation, or a combination of the two [23,146]. Most epidemiological studies conclude that less than 10% of PD has a strict familial etiology [110]. This includes a small number of familial parkinsonian patients with polymorphisms in the α-synuclein gene [112] (reviewed by Lundvig et al., this issue) as well as patients with early onset PD that have recessive mutations in the Parkin gene, mapping to the long arm of chromosome 6 (6q25.2-q27). The pathogenicity of these proteins is discussed in a review by Burke [9]. Familial PD has also been associated with human chromosome 2p13 and 4p polymorphisms [38]. The PD linked to this locus more closely resembles that of idiopathic PD, although like the α-synuclein protein, this unknown protein has very low penetrance. Although alterations in the proteins coded for by these loci may lead to an understanding of the molecular processes that occur in idiopathic PD, no mutations as of yet have been reported in aged-onset idiopathic PD [56,138].

Since the majority of PD patients have no identifiable genetic mutation, important information regarding the pathophysiology of PD may be learned through the study of animal models. At this time, several animal models have been developed to study the underlying mechanisms that lead to the development of experimental PD. One of the earliest models made use of a lesion of nigrostriatal pathway in which fibers emanating from the substantia nigra proceeded to the striatum rostrally through the medial forebrain bundle, [6,31,80]. Other models have used chemical lesions. One example is the use of 6-OHDA, a neurotoxin that when injected into the striatum causes a retrograde degeneration of dopaminergic neurons in the SNpc (reviewed in Refs. [22,55,107,137]). Another model of experimental PD utilizes the properties of selective neurotoxins, including 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP).

The sequalae of degeneration that occurs following administration of MPTP in animals has provided a useful model of Parkinsonism because it induces pathologies similar to that seen in man. Interestingly, the use of MPTP may be one of the few cases in which the effects of a neurotoxin were discovered in humans first, followed by development of an animal model. Although there were early reports of neurotoxicity to this compound, its use as a research tool became prevalent in the early 1980s following the identification of a number of Northern California heroin users who presented at various emergency rooms with symptoms indistinguishable from those of Parkinson's disease [10,75]. A complete history of these cases is presented in "The Case of the Frozen Addicts" [76].

Although MPTP was first identified as a parkinsonian agent in humans, it has been demonstrated to exert similar effects in a number of other primates [63,70,157], as well as in cats, and in several rodents. In rodents, it has been shown that only specific strains of mice are sensitive to the administration of MPTP [46,94,123,148]. MPTP structurally resembles a number of known environmental agents, including well-known herbicides, such as paraquat [24], and garden insecticides/fish toxins, such as rotenone [90], that have been shown to induce dopamine cell degeneration [4,8,13,153], although mechanistically, the actions of each are likely different [24]. Further studies of each of mechanism of each of these toxins may lead to a unified pathway that underlies these toxins.

2. Mechanism of MPTP action

There are many points systemically where MPTP can affect the dopaminergic system (Fig. 1). The first point of potential modulation of any exogenous compounds neurotoxicity is the blood–brain barrier (BBB). The BBB is composed of tight-junctioned endothial cells that make up the microvasculature of the brain in tight opposition with the end feet of glial processes. MPTP first is metabolized by the enzyme MAO-B to 1-methyl-4-phenyl-2, 3-dihydropyridium ($MPDP^+$) that then deprotonates to generate the corresponding pyridium species, MPP^+. Endothelial cells in the microvasculature that make up the BBB contain monoamine oxidases; and several studies have correlated levels of monoamine oxidases with MPTP-induced neuronal loss [64,124]. Since MPP^+ cannot be transported through the BBB [125], this level of toxification/detoxification can provide a first line of defense against exogenous agents.

3. Role of glial cells (part 1)

MPTP that is not converted to MPP^+ in the periphery rapidly enters the brain where it is processed into glial cells by a number of mechanisms, including monoamine [7] and glutamate [50] transporters or pH-dependent antiporters [69,87]. Glia, like the previously mentioned endothelial cells, also contain large pools of monoamine oxidases, and also convert MPTP from its protoxin form to MPP^+ [122]. Additional support for the role of glial cells in dopaminergic neuronal toxicity was shown by Brooks et al. [7] who demonstrated that administration of a serotonergic uptake inhibitor, fluoxetine, immediately before systemic injection of MPTP altered the observed neurotoxicity. Since fluoxetine did not alter the neurotoxicity of injected MPTP, it was proven that the site of activation was extraneuronal, lending credence to the observation that the primary step in MPTP toxicity occurred in the astrocyte.

Once converted to MPP^+ in the astrocyte, MPP^+ stimulates the up-regulation of TNF-α, interleukin-1β (IL-1β) and IL-6 [152,160] and these, in turn, up-regulate inducible nitric oxide synthase (iNOS) [57]. Of the three NOS isoforms present in the brain, endothelial NOS

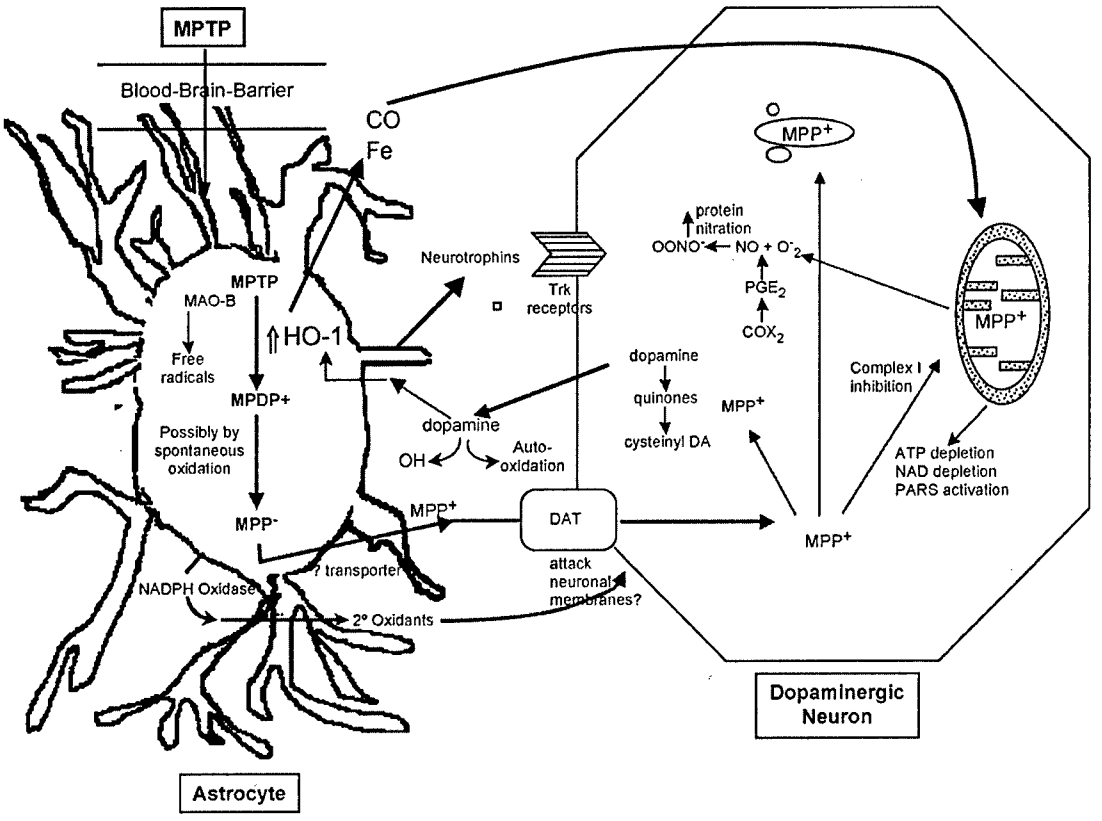

Fig. 1. Schematic representation of the mechanism of MPTP action in the nigrostriatal system. Red arrows represent the initial (toxification) role of glial cells. Blue arrows represent the second (neuroprotective) role of glial cells.

(eNOS), found mainly in the vasculature of the brain, is not altered following to MPTP toxicity [98]. In addition, since neuronal NOS (nNOS) knockout mice show partial protection against the MPTP toxicity in the substantia nigra pars compacta (SNpc) [116], another NOS isoform must also contribute to the neurotoxicity of MPTP. iNOS, a NOS isoform that is minimally expressed in the normal brain, has been shown to be up-regulated in the substantia nigra's microglia in both Parkinson's disease (PD) and in mice following MPTP treatment [68,81,158,159]. iNOS produces large amounts of the uncharged and lipophillic molecule nitric oxide (NO) and as such can freely pass through membranes and travel distances greater than the length of a neuron [73]. Thus, following MPTP treatment, neurons in the vicinity of the NO molecule are put at risk for possible attack by glial-derived reactive nitrogen-related species. Since iNOS induction can be blocked by the antibiotic minocycline [158], this step in the toxification process of MPTP presents a point where potential therapeutics may have a significant impact.

Since MPP^+ is a polar compound, it cannot freely exit from its glial environs. It has been suggested that there may be a specific transporter that actively moves this polar molecule out of the glia [58,127]; however, at present, this specific mechanism remains unknown.

Once MPP^+ is released into the extracellular space, MPP^+ is taken up into dopaminergic cells by the dopamine transporter (DAT). Since midbrain neurons contain the highest concentration of dopamine transporters/cell [11], the DAT may be a control point in determining how susceptible midbrain neurons are to exogenous agents [67,79] (but see also Ref. [52]). The requirement for the DAT in relation to MPTP toxicity was demonstrated by two groups examining mice carrying null mutations of the DAT [5,36]. In these studies, MPTP-susceptible strains of mice carrying null mutations of the DAT were completely protected from MPTP toxicity.

4. Role of dopaminergic neurons

Once in the cell, MPP^+ can move through several cellular compartments: it can enter into mitochondria where it interferes with complex I of the electron transport chain [74,103] or it can be sequestered into cytoplasmic vesicles by actions of the vesicular monoamine transporter [20,82].

MPP^+ enters the mitochondria by the diffusion through the mitochondrial inner membrane. The uptake of MPP^+ into mitochondria is actively driven by a membrane electrical gradient ($Km \approx 5$ mM). This active transport was supported by experiments in which valinomycin plus potassium, which collapses the mitochondrial electrochemical gradient, eliminated MPP^+ uptake, while agents which

collapsed this proton gradient had no effect on MPP^+ uptake [120,121].

Once in the mitochondria, MPP^+ inhibits cellular respiration through the blockade of the electron transport enzyme NADH:ubiquinone oxidoreductase (complex I) [102,149]. Blockade of this complex leads to a reduction in cellular ATP. Although this appears to be the major step in blockade of mitochondrial function, studies have shown that MPP^+ can also directly inhibit complexes III (ubiquinol:ferrocytochrome c oxidoreductase) and IV (ferrocytochrome *c*:oxygen oxidoreductase or cytochrome *c* oxidase) of the electron transport chain [95,96]. The loss of cellular energy through each of these pathways has several consequences, including the generation of the oxygen free radicals that rearranges to form hydrogen peroxide. Further catalysis leads to the formation of hydroxyl radicals.

Based upon the finding that MPP^+ depletes cellular energy due to interference with complex I–III, and as such may be related to the etiology of human PD, a number of potential therapies have been examined. One promising study has used Coenzyme Q10 supplementation, where oral administration of this compound in fairly high doses has been observed to slow the progression of the disease [2,97,139].

Although complex I inhibition by MPP^+ reduces energy production within dopaminergic neurons, it is likely that this is not the immediate cause of the SNpc neuronal death. The damage done within these dopaminergic neurons is likely to result from compounds generated in the cell, secondary to energy depletion. The formation of the superoxide radical is one example of this process. Cleeter et al. [14] showed that MPP^+, following inhibition of mitochondrial complex 1 activity, formed an excessive amount of superoxide radicals within the neuronal cytosol. Further support for the role of superoxide radicals came from Przedborski et al. [115], who demonstrated that overexpression of the copper–zinc form of superoxide dismutase in mice is neuroprotective against the damaging effects of MPTP. Moreover, Wu et al. [159], using the fluorescent tag hydroethidium, provided an in vivo demonstration of the presence of the superoxide radicals following MPTP intoxication.

NO, produced and released by glial cells, can enter the cytosol of the neuron via simple membrane diffusion. At this point, the superoxide radical and NO, which are not particularly damaging by themselves; can interact to form peroxynitrite ($OONO^-$), one of the most destructive oxidizing molecules [59,114,117]. Although difficult to detect due to its rapid processing, the nitration of the tyrosine residues of a number of cellular components that include enzymes, transmitters, proteins, fatty acids and DNA can easily be identified [119].

One potential target of $OONO^-$ is tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine synthesis. The most densely packed TH-positive cell area in the brain is the SNpc, which projects fibers to the striatum [44]. Since it has been shown that the cell-body rich SNpc primarily contains the soluble form of the TH enzyme, it is often used as the phenotypic marker for dopaminergic neuron numbers and can be measured both by biochemical and immunohistochemical methods to determine neuron loss [46,60].

Clinical symptoms are first thought to appear when about 60–70% of the TH-positive cells in the SNpc have degenerated [30]. Brains of parkinsonian patients also show deficits in TH enzyme and TH enzyme activity [61]. Both in vitro and in vivo studies demonstrate that peroxynitrite impairs TH activity [71]. In MPTP-treated mice, TH nitration occurs soon after MPTP administration. Furthermore, transgenic mice that overexpress human SOD do not show any detectable levels of nitrated striatal TH following MPTP treatment [1]. Mice deficient in iNOS show less ventral midbrain nitrotyrosine, a fingerprint for tyrosine nitration, after MPTP administration than in their wild-type counterparts [81]. Thus, the inactivation of TH via its nitration following exposure to both peroxynitrite and MPTP appears to be an important process in the development of PD in humans and to the MPTP neurotoxic process in mice.

Dopamine (DA) is a relatively unstable molecule that is subject to both hydroxyl radical attack [141] and autooxidation in the extracellular space [53]. In addition to extraneuronal effects, dopamine can also be nitrated intracellularly [78] and therefore may contribute to the degeneration of the cells that contain this neurotransmitter. In this process, DA is oxidized to DA quinone, which then undergoes a nucleophilic addition via the transfer of a sulfhydryl group from cysteine, to form 5-cystenyl-DA [43]. In pathological situations, the oxidation of DA to 5-cystenyl-DA is facilitated by the up-regulation of cyclooxygenase-2 (COX-2) [48,105]. The role of 5-cystenyl-DA in the development of PD as well as to the degeneration of DA neurons seen in the MPTP mouse model was examined in a variety of studies. For example, it was observed that COX-2 immunostaining was robust in the human and mouse dopaminergic neurons [151]. Additionally, COX-2 enzyme activity and protein levels in both PD brains and ventral midbrain from MPTP-treated mice were found to be significantly higher than in controls. Inhibition of the COX-2 response to MPTP, however, prevented the rise in protein cysteinyl dopamine that was seen to occur in mice following the administration of MPTP [151].

The vesicular monoamine transporter VMAT2, is a proton-dependent transporter that sequesters monoamine neurotransmitters from free cytoplasmic space into synaptic vesicles [93]. Since it structurally resembles monoamines, MPP^+ can be transported by the VMAT into these vesicles, thus being prevented from entering the mitochondria where it can inhibit complex I. This sequestration has been hypothesized to be as a potential mechanism for reducing the deleterious effects of any number of monoaminergic toxins. Support for this hypothesis comes from analyses in mice containing partial or complete deletions of VMAT2 as well as from human studies of VMAT expression. In parkinsonian humans, immunocytochemical localization of

VMAT showed reduced expression in striatum, similar to that seen in the DAT. In fact, Miller et al. [92] suggested that the relative expression of VMAT2, compared to that of DAT, may allow one to predict if and which dopamine neurons may be lost in PD (see the possible application of this hypothesis in Faherty et al., this issue). In animal studies, VMAT2(±) mice exposed to MPTP were examined for markers of dopaminergic neuron toxicity, including dopamine content and DAT protein in the stiatum, as well as expression of glial fibrillary acidic protein (GFAP) mRNA. In all parameters measured, VMAT2(±) mice were more sensitive than their wild-type littermates to MPTP-induced toxicity [37]. Further examination of these mice revealed that VMAT2(±) mice, following administration of MPTP, also had increased SNpc cell loss [150]. These studies suggested an important role for VMAT2 in potentiating the effects of MPTP. Using an in vitro system, cells transfected to overexpress a greater density of VMAT2 were converted from MPP^+-sensitive to MPP^+-resistant cells [82]. These studies suggested an important role for VMAT2 in modulating the effects of MPTP. Another molecule of interest relevant to the development of PD in humans and to the neurotoxic process in the MPTP mouse model of PD is α-synuclein. Synucleins are cytosolic proteins that contain 127–140 residues that have a unique 11-residue repeat that occurs in 5–7 copies which accounts for roughly one-half of their structure and no structural domains (see review by Lundvig et al., this issue). Four proteins, α, β and γ synuclein as well as synoretin make up this family of proteins of which only two, α and β, are synthesized in relatively large amounts in the brain (making up approximately 1% of total brain protein). These proteins are generally found in abundance in presynaptic nerve terminals [134]. Mutations in α-synuclein have been associated with a familial form of PD [112] that is readily indistinguishable from the more common sporadic form of the disease. It is thought that the interaction between WT and mutant α-synucleins may enhance the ability of these proteins to interact with other non-synuclein cellular proteins to form aggregates [15].

The presence of Lewy bodies within neurons in the SNpc is one of the characteristic pathologies seen in PD. Lewy bodies are both ubiquitin and α-synuclein immunopositive. Since α-synuclein is the only synuclein present in Lewy bodies, it has to be determined whether this molecule is toxic or whether it is just a by-product (tombstone) of cellular metabolism in a pathological situation. A number of cellular proteins have been found to be nitrated in PD brains [59] and specific antibodies that recognize nitrated α-synuclein have been used to demonstrate that alpha-synuclein is the protein that is nitrated in Lewy bodies [40,41]. Furthermore, α-synuclein inclusions in tissues from PD patients have been shown to be strongly labeled with antibodies that recognize the hallmark of peroxynitrite-induced nitration, 3-nitrotyrosine [144]. Two lines of evidence support these conclusions. In vitro studies using HEK 293 cells transfected to overexpress human α-synuclein that were exposed to peroxynitrite showed a nitrated band that corresponded to the molecular mass of α-synuclein was noted [118]. In vivo, using the MPTP mouse model, immunoprecipitation studies of midbrain and striatum showed that α-synuclein was nitrated as early as 4 h after MPTP administration. Specificity for this form of synuclein was demonstrated by the observation that β-synuclein was not nitrated in either situation [118].

Another consequence of the cellular ATP depletion is the abnormal release of DA from intracellular stores [77,106,126,135,136]. Once DA is released into the extracellular space, the enzymatic oxidation of DA results in the formation of hydroxyl radicals. That dopamine rapidly auto-oxidizes and contributes to neurotoxicity always leads to the controversial topic of L-dopa therapy in PD. Simply stated, one can question whether the therapy that best treats the symptoms of PD may also exacerbate the disease. In support of this hypothesis, Whone et al. showed that the progression of PD using PET scanning was greater in patients treated with L-dopa than those treated with the dopamine agonist ropinerole [86,156]. However, other studies do not support this hypothesis [33,91], and for this reason, this question has yet to be sorted out.

Additional sites of hydroxyl radical formation may occur as a result of interactions with neuromelanin [17] as well as with cellular iron [62], each of which could contribute to its neurotoxicity.

5. Role of glial cells (part 2)

The mechanism(s) of MPTP-induced cell death (Fig. 1) show a great amount of cross-talk between the neurons and the nonneuronal milieu. Previously, we discussed how the astrocytes are necessary for the conversion of MPTP to MPP^+. In addition to this function, astrocytes are also believed to play a significant role in neuroprotection. A study using chimeric SN cell cultures, has demonstrated that the differential toxicity of MPTP in mouse strains is determined by the response of the glial cells [142]. This work is supported by other in vitro studies [25,26,34].

As discussed earlier in this review, glial cells directly contribute to the toxicity seen following administration of MPTP through several mechanisms, including the mediation of free radical formation and damage by induction of nitric oxide synthase (iNOS) [54,88,89]. In addition to the induction and modulation of cytokines, the presence of dopamine in the extracellular space can induce a number of different molecules that are involved in oxidative stress. One of these molecules, hemeoxygenase-1 (HO-1), the rate-limiting enzymes in heme degradation, has been shown to play a critical role in iron and heme homeostasis [85,133]. It is well known that alterations in brain iron are seen in PD brains [3,65,72]. Several isoforms of hemeoxygensase have been identified (reviewed in Ref. [29]), each of which converts

heme to bilirubin and carbon monoxide, while at the same time releasing iron into the cellular milieu [84]. Further support for the importance HO-1 is that it is elevated in astrocytes of parkinsonian patients [132]. In striatal astrocytes, HO-1 elevation occurs as early as 6 h following administration of MPTP [32]. In addition, brains of HO-1 null mice show excessive deposition of iron, increased sensitivity to oxidative stress and chronic inflammation [113]. On the flip side, overexpression of HO-1 leads to a lessening of damage that has been observed in the presence of free radicals [84]. For this reason, modulation of HO-1 has been postulated as a potential therapy for PD. However, based on the breakdown of heme, which leads to the formation of bilivirdin, carbon monoxide and free iron, it is possible that in the specific environment of the SN, HO-1 can act counterintuitively and lead to a furthering of neurotoxicity [47,131]. It is also possible that the breakdown products of heme induced by HO-1 act as mitochondrial toxins leading to a feed-forward loop that eventually leads to cell death.

In addition to acting as participants in cellular toxicity, astrocytes, either in the substantia nigra or striatum, may also act as a protective agent through several mechanisms, including their ability to act as a "cellular buffers" and production of neurotrophic factors. Several studies have shown that astrocytes synthesis and release the free-radical scavenger glutathione and/or its precursors glutamate, cysteine and glycine [27]. This function is specific to astrocytes and not neurons—as they are able to generate this neuroprotectant thorough the biochemical pathways that use cystine as well as cysteine for the production of GSH (Sagara, 1993 #1117, [28,155]). Since GSH levels are lower in the SNpc of PD patients, the local SNpc astrocytes may serve this critical function. The efficiency of glial cells in producing or in maintaining levels of glutathione in different strains of mice [49] may be an important factor in the pathogenesis of dopaminergic neuron loss in experimental models of PD and may point to this pathway as a therapeutic target for neuroprotection.

In addition to providing the precursors for redox-modulating compounds, such as glutathione, astrocytes have also been shown to produce a number of neurotrophic factors [101,128,129]. Several neurotrophins have been shown to protect dopaminergic neurons from cell death following MPTP or MPP^+ intoxication [100]. These factors include BDNF [35,145,154], GDNF [12,19], FGF [108] and EGF [45]. Neurotrophins act to prevent cell death through a number of mechanisms including modulation of oxidative stress [42,66,111,140,145] as well as interference with the intrinsic cell death programs [51,130].

6. Conclusions

MPTP, which is structurally similar to a number of commonly used herbicides and pesticides, can induce specific loss of substantia nigra neurons in many vertebrate species, from man to mouse. Studies using this toxin have led to the development of useful animal models of Parkinson's disease. In mice, MPTP demonstrates differential toxicity that is dependent on the strain of animal examined [46,99,143] (see also Pierri et al. in this issue). This finding supports the hypothesis that the loss of substantia nigra neurons in Parkinson's disease may result from a genetic sensitivity to a number of environmental agents [24,147]. Recently, the chromosomal loci containing the genetic sequences contributing to this sensitivity to neuronal loss [16] in mice has been localized on the telomeric end of mChr.1 [16]. Further studies into the genetic as well as into the biochemical pathways involved in MPTP toxicity will lead to a better understanding of idiopathic Parkinson's disease as well as provide clues to novel targets for therapeutic interventions.

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MPTP and SNpc DA Neuronal Vulnerability: Role of Dopamine, Superoxide and Nitric Oxide in Neurotoxicity. Minireview.

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Parkinson disease (PD) is a common neurodegenerative disease of unknown origin that is characterized, mainly, by a significant reduction in the number of dopamine neurons in the substantia nigra pars compacta (SNpc) of the brain and a dramatic reduction in dopamine levels in the corpus striatum. For reasons that we do not know, the dopamine neuron seems to be more vulnerable to damage than any other neuron in the brain. Although hypotheses of damage to the dopamine neuron include oxidative stress, growth factor decline, excitotoxicity, inflammation in the SNpc and protein aggregation, oxidative stress in the nigrostriatal dopaminergic system garners a significant amount of attention. In the oxidative stress hypothesis of PD, superoxide, nitric oxide and dopamine all conspire to create an environment that can be detrimental to the dopamine neuron. MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), the tool of choice for investigations into the mechanisms involved in the death of dopamine neurons in PD, has been used extensively in attempts to sort out what happens in and around the dopamine neuron. Herein, we review the roles of dopamine, superoxide and nitric oxide in the demise of the dopamine neuron in the MPTP model of PD as it relates to the death of the dopamine neuron noted in PD.

Keywords: MPTP; Substantia nigra pars compacta; Neurotoxicity; Dopamine; Superoxide; Nitric oxide; Oxidative stress; Parkinson's disease

INTRODUCTION

Most neurodegenerative diseases involve specific subsets of neurons. In the case of Parkinson's disease (PD), a common neurodegenerative disorder characterized behaviorally by resting tremor, rigidity, akinesia/bradykinesia and postural instability, these are mainly, though not exclusively, the dopaminergic neurons in the substantia nigra pars compacta (SNpc) whose fibres project to the corpus striatum. There are, at present, 1 million PD patients in the United States alone, with 50,000 newly diagnosed cases each year (Fahn and Przedborski, 2000). These cases include both familial and sporadic PD, of which sporadic PD appears to be the more common (Dauer and Przedborski, 2003). Currently, the most effective therapy for alleviating the symptoms of PD is levodopa (L-DOPA) (Fahn and Przedborski, 2000), which increases the levels of dopamine in the brain. Although, there is no evidence that L-DOPA alters the progression of the disease, on one hand, speculations exist that L-DOPA may actually contribute to the progression of PD (Fahn, 1997; Weiner, 2000) while on the other, it is thought that L-DOPA is actually neuroprotective (Rajput, 2001) and non-toxic to the human substantia nigra (Rajput, 2001). For reasons that are not yet understood, dopaminergic neurons in the SNpc appear to be more susceptible to damage than other neurons in the brain. Theories as to why this situation exists include genetics (Vila and Przedborski, 2004), excitotoxicity (Olanow and Tatton, 1999), inflammation in

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the brain due to changes in the neuronal environment (Langston *et al.*, 1999; Hunot and Hirsch, 2003, Teismann *et al.*, 2003), protein aggregation (Ii *et al.*, 1997; Trojanowski *et al.*, 1998) oxidative stress, (Fahn and Cohen, 1992; Przedborski and Jackson-Lewis, 2000), and growth factor (neurotrophin) decline (Mogi *et al.*, 1999; Nagatsu *et al.*, 2000).

About 10% of the PD cases are familial. To date, a number of genetic mutations have been found both in multiple pedigrees and in single families. Multiple pedigree mutations include those found in the alpha-synuclein, parkin, Nurr-1 (nuclear receptor related-1) and DJ-1 genes whereas UCHL-1 (ubiquitin c-terminal hydrolase-1) and NF-M (neurofilament medium) gene mutations have been localized to single families (Huang *et al.*, 2004). Most, if not all of these identified genes function for the survival of the dopamine neuron (synthesis, metabolism, energy supply, cellular detoxification). Thus, any mutation in these genes could lead to misfunctions in the dopamine neuron making them more susceptible to such problems as energy crisis and oxidative stress that could lead to eventual death. In addition to these PD-inducing mutations, several mechanisms have been proposed regarding the etiology of PD. These include ion homeostasis, neuroinflammation, protein aggregation and alterations in growth factors.

Calcium homeostasis is important to normal dopamine neuron function. The NMDA (*N*-methyl-D-aspartate) and the AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate) receptors, both ionotrophic glutamate receptors, are, in part, responsible for intracellular calcium homeostasis (Rego and Oliveira, 2003). Overstimulation of these glutamate receptors can alter local calcium homeostasis. Calcium is known to up-regulate enzymes like phospholipase A_2, nitric oxide synthase and xanthine oxidase, all of which are found in mitochondria and all can stimulate reactive oxygen species (ROS) production. Thus, if local calcium control is compromised, resulting in an excitatory-stimulated release of ROS and if existing antioxidant systems cannot handle the produced ROS, mitochondrial dysfunction and damage to several synaptic and intracellular proteins ensues.

Progression of a number of neurological diseases has been shown to be related to inflammation in the brain, which can affect the neuronal environment. For instance, multiple sclerosis is a neuroinflammatory disease that causes a loss of the myelinated tracts in the CNS (Hafler, 2004), and recent evidence has shown that there is an inflammatory component to amyotrophic lateral sclerosis (Drachman *et al.*, 2002). Furthermore, supporting a role for inflammation in PD is the finding by Langston and colleagues that brains from individuals, who died from a PD-like syndrome resulting from the self-administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and who lived for 3-16 years following exposure, showed a significant presence of activated microglia (Langston *et al.*, 1999). However, inflammation, thus far, has not been shown to be the cause of PD but rather it is suggested that inflammation may be instrumental in its progression.

Although there is still much debate on the subject, the finding of protein aggregates (both intracellular and extracellular) in many of the neurodegenerative diseases including PD has led to the hypothesis that the improper disposition of proteins may be toxic to the dopamine neuron and can contribute to the neurodegenerative process. In the simplest of terms, the ubiquitin-proteasome system (UPS) is like a sink which degrades both abnormal and damaged proteins in the neuron. Proteins are first ubiquinated by the covalent attachment of a polyubiquitin chain and then the whole complex is degraded by the 26S proteasome (Vigouroux *et al.*, 2004). If this system fails to operate properly, it is thought that the aggregation of proteins to be disposed of follows. For example, Lewy bodies are a pathological hallmark of PD and they contain significant amounts of modified alpha-synuclein (Dauer and Przedborski, 2003). Recent reports have shown that aggregated alpha-synuclein not only binds to but also inhibits ubiquitin-dependent proteasomal function (Snyder *et al.*, 2003). Furthermore, oxidized proteins can accumulate in the neuron and this abnormal accumulation of proteins may be toxic enough to put the neuron in an oxidative stress situation which is a highly damaging event.

The growth factor decline hypothesis begs the question as to why these substances are decreased in the SNpc of PD brains. Growth factors (neurotrophins) are proteins that are normally highly expressed in the substantia nigra (SN) and several lines of evidence demonstrate a decrease in growth factors, particularly glial-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) in the SN of PD brains (Chauhan *et al.*, 2001). The reason for these decreases remains unknown. And, there is nothing known about growth factor decline and oxidative stress. What is clear is that most of the aforementioned hypotheses involve some kind of oxidative stress situation. We and others have used MPTP to follow the oxidative stress hypothesis and the proposed roles of superoxide, nitric oxide and dopamine in the vulnerability of the dopamine neuron (FIG. 1).

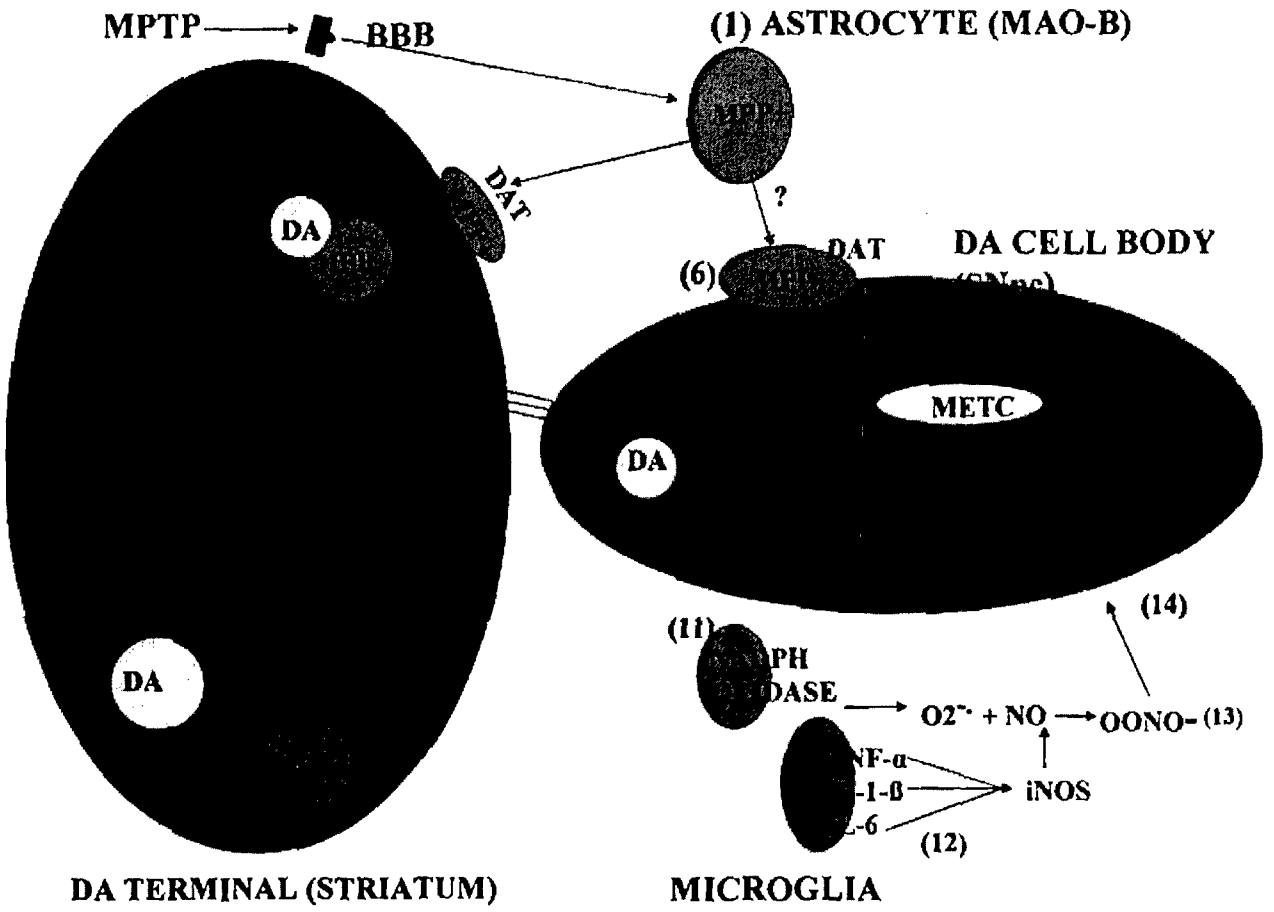

FIGURE 1 Proposed MPTP action in the nigrostriatal dopaminergic pathway. After systemic administration, MPTP freely crosses the blood-brain barrier and is taken up into astrocytes where it is metabolized by monoamine oxidase-B (MAO-B) to MPP (1). It then exits the astrocyte by an unknown mechanism and is taken up by the dopamine transporter (DAT) into the dopamine terminals in the striatum. There, MPP^+ is sequestered into the storage vesicles (2) and in the process pushes out dopamine (3) which is oxidized to either dopamine-*o*-quinone (dopamine-*o*-Q), 6-hydroxydopamine (6-OHDA), 3,4-dihydroxyphenylacetaldehyde (DOPAL) or 5-cysteinyldopamine (5-cysteinyl- dopamine) (4). Dopamine-*o*-Q (DA-*o*-Q), 6-OHDA and DOPAL have all been shown to be toxic and can attack dopamine nerve terminals (5) that lead back to the SNpc dopamine cell body. In the meantime, in the extracellular space in the SNpc, MPP^+ that is extruded from astrocytes, is taken up into the neuron by the DAT (6). Once in the cell body (7), MPP^+ blocks the complex 1 site of the mitochondrial electron transport chain (METC) which causes the METC to kick out the superoxide radical. Neuronal nitric oxide synthase (nNOS) in the METC membrane up-regulates, which increases the presence of nitric oxide (NO). In the cytosol of the dopamine neuron, superoxide and NO interact to produce the strong oxidant peroxynitrite ($OONO^-$) (8) which can damage cellular proteins, lipids and DNA. Dopamine in the neuron is oxidized to dopamine-*o*-Q and DOPAL (9) or can be released and subjected to hydroxyl radical attack (6-OHDA?) (10) which can also damage the neuron. Once the neuron is damaged, an inflammatory response ensues in which microglia in the extracellular space become activated. During the activation process, certain enzymes are up-regulated [NADPH oxidase (11), tumor necrosis factor (TNF)-α, interleukin-1-beta (IL-1-ß), IL-6 and inducible NOS (iNOS) (12)] which stimulate superoxide and NO production. These two then react to produce OONO (13) which can attack the neuronal membrane (14).

The MPTP Neurotoxic Process

As a highly lipophilic compound, MPTP can be absorbed through the skin, be ingested, injected, and snorted. However administered, MPTP rapidly crosses the blood brain barrier and is taken up into glial cells by monoamine (Brooks *et al.*, 1989) and glutamate transporters (Hazell *et al.*, 1997) or pH-dependent antiporters (Kopin *et al.*, 1992; Marini *et al.*, 1992). Once inside glial cells, MPTP is converted to $MPDP^+$ (1-methyl-4-phenyl-2,3-dihydropyridinium) by monoamine oxidase-B (MAO-B) and then to MPP^+ (1-methyl-4-pyridinium) (Ransom *et al.*, 1987) by spontaneous oxidation. Since MPP^+ is a polar compound, it cannot cross membranes; it is speculated that MPP^+ is extruded from glia via some kind of transport system. Evidence for a role of glia in the conversion of MPTP

to MPP^+ comes from Brooks *et al.* (1989) who demonstrated that fluoxetine, a serotonin uptake inhibitor, attenuates MPTP-induced dopaminergic toxicity but does not interfere with MPTP metabolism. Following extrusion into the extracellular space, MPP^+ is taken up into the dopamine neuron by the dopamine transporter (DAT) (Kostic *et al.*, 1996). This transporter may be damaged in the MPP^+ uptake process, as recent evidence by Jakowec *et al.* (2004) have shown that the number of DAT in the SN following MPTP administration is decreased. MPTP targets primarily dopamine neurons and the syndrome it produces, over a period of about a week (Jackson-Lewis *et al.*, 1995), is reminiscent of end-stage PD. MPTP causes a far greater loss of dopamine neurons in the SNpc than of those dopamine neurons in the ventral tegmental area. It also produces about 90% degeneration of dopamine nerve terminals in the striatum (Jackson-Lewis *et al.*, 1995). In order for this level of damage to occur in the nigrostriatal dopaminergic pathway, MPP^+ has to stimulate or recruit dopamine-related metabolites from within this pathway.

MPTP and Glial Cells

The non-neuronal support system in the CNS are the glial cells (Abbott, 1988). Under physiological conditions, glia secrete substances into the extracellular environment that support the normal functioning of the neuron (Abbott, 1988). For instance, not only is it known that microglia remove debris from the neuronal environment but, depending on the situation, they can be a source of neurotrophic and neuroprotective molecules such as interleukin-6, basic fibroblast growth factor, epidermal growth factor, insulin-like growth factor and epidermal growth factor. On the other hand, they can also produce neurotoxic compounds like nitric oxide, superoxide, tumor necrosis factor, glutamate, arachidonic acid and proteolytic enzymes (Banati *et al.*, 1993). Astrocytes seem to exert a protective effect on dopaminergic neurons, as it has been demonstrated that they can produce neurotrophins like nerve growth factor, ciliary growth factor and interleukin-6 (Muller *et al.*, 1995) as well as GDNF (Bohn, 1999). What is extremely interesting about these glial cells is that they may represent a double-edged sword when it comes to MPTP, for in the MPTP neurotoxic process, it is in glia that MPTP is metabolized to MPP^+ by MAO-B, and microglia produce molecules such as the superoxide radical and nitric oxide which are toxic to dopamine neurons.

MPTP, Superoxide and the SNpc Environment

The environment surrounding SNpc neurons can control the fate of these cells. For example, following MPTP administration, both the extracellular and the intracellular environments of the SNpc dopamine neuron are altered in such a way that they are no longer part of a supportive system but rather contain detrimental components. Our early studies using transgenic mice that overexpress the copper-zinc form of superoxide dismutase (CuZnSOD) and that were treated with MPTP show that the SNpc of these mice was protected against the damaging effects of MPTP (Przedborski *et al.*, 1992), thus implying the involvement of the superoxide radical. Furthermore, Wu *et al.* (2003) have shown that the infusion of SOD1 into the striatum of MPTP-treated mice is neuroprotective to SNpc neurons, which defines a role for the superoxide radical in the MPTP neurotoxic process. Since CuZnSOD is an extracellular enzyme (Fridovich, 1995), these results suggest that the extracellular environment of the dopamine neuron is perturbed or altered by the superoxide radical early in the neurotoxic process.

A significant source of the superoxide radical in the extracellular environment is NADPH oxidase (Gao *et al.*, 2003; Wu *et al.*, 2003). NADPH oxidase is a multimeric microglial enzyme that is composed of a number of subunits that include $gp91^{phox}$, $p22^{phox}$, $p47^{phox}$, and $p40^{phox}$ (Babior, 1999). In resting microglia, this enzyme is inactive because $gp91^{phox}$ and $p22^{phox}$ are separated from the other phox subunits. However, following MPTP administration to mice, the NADPH oxidase complex within the microglia becomes activated because the p47 phox subunit is phosphorylated followed by the movement of the whole complex to the microglial membrane where it assembles with $gp91^{phox}$ and $p22^{phox}$. This makes the NADPH oxidase complex able to stimulate the production of the superoxide radical. Wu *et al.* (2003), using hydroethidium injections in MPTP-treated mice, visualized the presence of the superoxide radical within microglia located in the SNpc environment of these mice. Up-regulation of NADPH oxidase in postmortem SNpc tissues from PD brains was also shown (Wu *et al.*, 2003). The superoxide radical is then extruded into the extracellular environment where its presence not only alters the neuronal environment but also stimulates the production of secondary oxidants (Babior, 1999) which can, in turn, influence the integrity of the dopamine neuronal membrane, enter the dopamine neuron and affect its internal environment.

Dopamine neurons, as abundant as they are in the SNpc, are likely a victim of their own environment. Once MPP^+ exits the glial cells, it is taken up from the extracellular space into the dopamine neuron via the DAT (Javitch *et al.*, 1985; Bezard *et al.*, 1999).

Although recent evidence shows that these transporters are injured during the uptake process (Jakowec *et al.*, 2004), enough of them remain to transport MPP^+ into the cytosol of the dopamine neuron. DAT are absolutely necessary for the MPTP neurotoxic process as several groups (Gainetdinov *et al.*, 1997; Bezard *et al.*, 1999) have shown that MPTP does not harm mice lacking DAT. In the cytosol of the dopamine neuron, when MPP^+ is not taken up into the vesicles, MPP^+ can assist in altering the internal environment of the dopamine neuron by blocking the mitochondrial electron transport chain (METC) at the complex I site (Nicklas *et al.*, 1985; 1987). The major organelle within the dopamine neuron that produces the lion's share of superoxide radicals is the mitochondrion (Beal, 2003). This organelle controls oxidation-reduction reactions and is a major source of cellular energy through its oxidative phosphorylation reactions (Przedborski and Jackson-Lewis, 2000). At the complex I site of the METC, the superoxide radical is released into the cytosol where, under physiological conditions, it is controlled by the manganese form of SOD (MnSOD), which is located in the internal membrane of the mitochondrion (Keller *et al.*, 1998). Many investigators have found a decrease in complex I in various tissues including brain tissue from PD patients (Mizuno *et al.*, 1989; Shapira, 1990). Thus, low activity of complex I in the METC translates to increased production of superoxide radicals, a depletion of MnSOD and an oxidative stress within the dopamine neuron. An overabundance of superoxide radicals, as stimulated by the presence of MPP^+, apparently can no longer be controlled by MnSOD. Klivenyi and colleagues (Klivenyi *et al.*, 1998) have shown that, as long as sufficient stores of MnSOD are present: 1) mice are protected against the damaging effects of MPTP; and 2) the superoxide radical influences the internal environment of the dopamine neuron. Furthermore, MPP^+ has also been shown to affect complex III (Mizuno *et al.*, 1988), such that the increased production of the superoxide radical here also contributes to the disruption of the normal cytosolic environment within the SNpc dopamine neuron. The relevance of this particular scenario to PD is not well understood because it is not clear whether the deficit in complex I is or is not a cause of PD.

MPTP, Nitric oxide and the SNpc Environment

Nitrative stress related to NO has been documented in PD brains through demonstration of the presence of the inducible form of nitric oxide synthase (iNOS) (Hunot *et al.*, 1996; 1999) and has been tied, in part, to the activated glia in the vicinity of SNpc dopamine neurons. Evidence of the up-regulation of iNOS in glia following MPTP administration points to an indirect process rather than a direct up-regulation of this enzyme (Ciesielska *et al.*, 2003). In glia within the area of the SNpc and the striatum, MPP^+ stimulates the up-regulation of proinflammatory cytokines such as TNF-α, interleukin-1-beta (IL-1ß) and interleukin-6 (IL-6) (Youdim *et al.*, 2002; Teismann *et al.*, 2003) in a time-dependent manner (Hebert *et al.*, 2003) as early as 12-18 hours prior to the induction of iNOS (Hunot *et al.*, 1999). Immunohistochemical studies (Liberatore *et al.*, 1999; Dehmer *et al.*, 2000) show that iNOS up-regulation occurs in microglia 24 hours after the administration of MPTP, which suggests that the proinflammatory cytokines may stimulate the up-regulation of the iNOS enzyme and thereby increase the production of NO within the glia. In a personal communication, Wu and Przedborski (Personal Communication) noted that endothelial NOS (eNOS) is found in the brain vasculature and does not contribute to the MPTP neurotoxic process. In contrast, neuronal NOS (nNOS), shown to be decreased within non-dopamine neurons (? interneurons) in the SNpc following MPTP administration (Watanabe *et al.*, 2004), probably does contribute to the MPTP neurotoxicity in the SNpc. Furthermore, since nNOS knockout mice were only partially protected against the damaging effects of MPTP and 7-nitroindazole, a selective inhibitor of nNOS that has little if any cardiovascular effects, offered a greater but not a total protection (Przedborski *et al.*, 1996), it is likely that nNOS is a contributor to NO presence in the extracellular space and to the alterations in the extraneuronal environment of the dopamine neurons in the SNpc.

NO is not a free radical, is highly lipophilic, can readily traverse membranes without the need of a transport system and has the ability to travel as far as 300 microns from its site of production (Lancaster, 1996). Under physiological conditions, both nNOS and iNOS produce significant amounts of NO that are ever present in the extracellular space while levels of the superoxide radical, constantly produced in many biological reactions within the brain, are kept in check by the abundance of SOD. In the pathology of PD and in the MPTP model, increased amounts of the superoxide radical and NO are pushed into the extracellular milieu surrounding the dopamine neuron. Here, they can react with each other at a faster rate than the superoxide radical can be dismutated by the extracellular CuZnSOD to produce the most damaging secondary oxidant peroxynitrite (Przedborski *et al.*, 2000). Peroxynitrite can damage neuronal membrane proteins and lipids (Przedborski *et al.*, 2000). Thus, the extracellular neu-

ronal environment of SNpc is disturbed or compromised and is no longer supportive for the dopamine neuron either in PD or in the MPTP model.

Although the superoxide radical does disturb the internal environment of the dopamine neuron, it is, by itself, not overwhelmingly toxic. In the internal milieu of the SNpc dopamine neuron, aside from affecting the METC, MPP^+ has been demonstrated to increase the expression of the cyclooxygenase-2 (COX-2) enzyme (Teismann *et al.*, 2003). COX-2 is the rate-limiting enzyme in the conversion of arachidonic acid to PGH_2 which is then further metabolized to PGE_2 (O'Bannion, 1999). The NO present in the SNpc dopamine neuron following MPTP administration most likely enters the dopamine neuron after having traveled some distance from its non-dopamine neurons in the SNpc that contain nNOS. When both the superoxide radical and NO are in excess in the internal milieu of the dopamine neuron after MPTP exposure, PGE_2 catalyzes the reaction between these two relatively mildly toxic compounds to produce the secondary oxidant peroxynitrite (Ischiropoulos and al-Mehdi, 1995; Przedborski and Vila, 2003) which again creates a severely hostile environment for the dopamine neuron. Peroxynitrite nitrates internal cellular components such as enzymes, fatty acids, proteins, lipids, amino acids and DNA (Radi *et al.*, 2002) of which one of the most important of these is the tyrosine hydroxylase (TH) enzyme. This enzyme is the rate-limiting enzyme in the synthesis of dopamine and is either down-regulated or damaged in PD and in the MPTP model such that the production of dopamine is severely compromised (Ara *et al.*, 1997).

Dopamine Toxicity and the SNpc Environment

The dopamine neuron in the SNpc may indeed be, at least in part, a contributor to its own death. Following MPTP administration, huge amounts of dopamine are released from intracellular stores into the extracellular space (Lau *et al.*, 1991; Schmidt *et al.*, 1999). Once released, dopamine is either enzymatically metabolized by monamine oxidase-B to 3,4-dihydroxyphenylacetic acid and in the process, the hydroxyl radical is kicked out (Burke *et al.*, 2004) or it auto-oxidizes to form a number of toxic compounds including 6-hydroxydopamine (Graham, 1978). 6-hydroxydopamine is a known neurotoxin that has been used extensively for animal models in PD research (Jeon *et al.*, 1995; Przedborski *et al.*, 1995). It has been demonstrated that this compound destroys striatal dopamine terminals which results in the death of SNpc dopamine neurons (Przedborski *et al.*, 1995). Interestingly, one of the findings in PD and in the MPTP model is that there is a greater loss of striatal dopamine nerve terminals than dopamine cell bodies in the SNpc (Fahn and Przedborski, 2000). This may be related to the huge release of dopamine from the storage vesicles caused by the uptake of MPP^+. Furthermore, although 6-hydroxydopamine has never been found in brain tissues from PD patients nor in brains from the MPTP model, one can speculate on the possibility that 6-hydroxydopamine or a similarly related compound may contribute negatively to the external environment that surrounds the dopamine neuron, since dopamine is susceptible to hydroxyl radical (secondary oxidant) attack (Cohen, 1984). A more interesting scenario, however, has been proposed with 3,4-dihydroxyphenylacetaldehyde (DOPAL). DOPAL is the intermediate dopamine metabolite that has been shown to be neurotoxic (Burke *et al.*, 2003). To demonstrate that it is DOPAL and not dopamine that is neurotoxic, Burke and colleagues (Burke *et al.*, 2003) injected varying concentrations of both compounds into the SNpc of rats. These researchers showed that DOPAL was 5-10 times more neurotoxic than dopamine. Thus, in the extracellular space, because MAO-B metabolizes dopamine to DOPAL (Fornai *et al.*, 2000; Burke *et al.*, 2004), dopamine via DOPAL, possibly contributes to changes in the extracellular milieu. DOPAL may also be the reason why dopamine terminals are severely damaged.

In the internal metabolism of the DA neuron, DA can be oxidized to dopamine-*o*-quinone and further to 5-cysteinyldopamine (Hastings, 1995). Aside from having a role in peroxynitrite formation through its stimulation of PGE_2, the COX-2 enzyme can facilitate the oxidation of dopamine which can damage protein-bound sulfhydryl groups (Hastings, 1995). Using HPLC analysis, Teismann *et al.* (2003) showed that MPTP administration elevates ventral midbrain 5-cysteinyldopamine, which is considered a stable modification of dopamine and evidence that the formation of dopamine-*o*-quinone has occurred. Dopamine-*o*-quinone can contribute to the upheaval of the internal neuronal environment through glutathione depletion and the inactivation of TH (Kuhn *et al.*, 1999). On the other hand, while dopamine is metabolized to DOPAL extraneuronally by MAO-B, within the neuron, DOPAL is formed by MAO-A (Burke *et al.*, 2004). Furthermore, DOPAL is the major metabolite of dopamine in the human brain (Burke *et al.*, 1999) and levodopa, the drug of choice in the treatment of PD, has been shown to elevate significantly levels of DOPAL in the brain (Fornai *et al.*, 2000). As stated earlier, DOPAL has been shown to destroy the dopamine neuron at concentrations much lower than dopamine

itself (Burke *et al.*, 2003). Whether MPTP can elevate DOPAL levels in the brain and mimic the death of dopamine nerve terminals as seen in PD remains to be determined.

CONCLUSIONS

Environment plays a significant role in the well-being of the dopamine neuron. Several cell types including glia and the compounds that these cells secrete work together to maintain an environment suitable for dopamine neuron survival. Yet, at the same time, these same cells and agents, when perturbed such as following MPTP administration, can contribute to the death of the dopamine neuron through reactions which alter their physiological concentrations in the SNpc, thus putting the dopamine neuron in a compromised (oxidative stress) situation. Interestingly, the major players in both environments are relatively the same as is their interplay. Thus, dopamine, superoxide and nitric oxide may all conspire to keep the dopamine neuron in a highly sensitive state, and when presented with a catalyst like MPTP, this sensitivity can shift to vulnerability.

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Neurobiology of Disease

Ablation of the Inflammatory Enzyme Myeloperoxidase Mitigates Features of Parkinson's Disease in Mice

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Parkinson's disease (PD) is characterized by a loss of ventral midbrain dopaminergic neurons, which can be modeled by the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Inflammatory oxidants have emerged as key contributors to PD- and MPTP-related neurodegeneration. Here, we show that myeloperoxidase (MPO), a key oxidant-producing enzyme during inflammation, is upregulated in the ventral midbrain of human PD and MPTP mice. We also show that ventral midbrain dopaminergic neurons of mutant mice deficient in MPO are more resistant to MPTP-induced cytotoxicity than their wild-type littermates. Supporting the oxidative damaging role of MPO in this PD model are the demonstrations that MPO-specific biomarkers 3-chlorotyrosine and hypochlorous acid-modified proteins increase in the brains of MPTP-injected mice. This study demonstrates that MPO participates in the MPTP neurotoxic process and suggests that inhibitors of MPO may provide a protective benefit in PD.

Key words: MPTP; Parkinson's disease; oxidative stress; inflammation; neuroprotection; nitrotyrosine

Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder characterized by disabling motor abnormalities, which include tremor, muscle stiffness, paucity of voluntary movements, and postural instability (Dauer and Przedborski, 2003). Its main neuropathological feature is the loss of the nigrostriatal dopaminergic neurons, the cell bodies of which reside in the substantia nigra pars compacta (SNpc) and nerve terminals of which extend to the striatum (Dauer and Przedborski, 2003). Except for a handful of inherited cases related to known gene defects (Vila and Przedborski, 2004), PD is a sporadic condition of unknown pathogenesis (Dauer and Przedborski, 2003). However, epidemiological studies suggest that inflammation increases the risk of developing PD (Chen et al., 2003), and experimental models of PD show that inflammatory oxidants modulate SNpc dopaminergic neuronal death (Liberatore et al., 1999; Gao et al., 2002; Wu et al., 2002, 2003). For instance, NADPH oxidase and inducible nitric oxide synthase (iNOS), which are major sources of inflammatory oxidants, are upregulated in damaged areas in both PD and the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of PD (Hunot et al., 1996; Liberatore et al., 1999; Wu et al., 2003). Studies of mice deficient in NADPH oxidase or iNOS indicate that superoxide radical (O_2^-) and NO contribute to the MPTP-induced neurodegenerative process (Liberatore et al., 1999; Wu et al., 2003). However, both O_2^- and NO are relatively unreactive, and a variety of secondary oxidants, such as peroxynitrite ($ONOO^-$), are more likely to account for the injurious capacity of inflammation in PD. Supporting this view are the demonstrations that levels of 3-nitrotyrosine, a major product of $ONOO^-$ oxidation of proteins, are elevated in affected brain areas after MPTP injections to mice (Pennathur et al., 1999), for the most part in an iNOS-dependent manner (Liberatore et al., 1999).

Levels of *O,O'*-dityrosine also increase markedly in the SNpc of MPTP-intoxicated animals (Pennathur et al., 1999). This is an intriguing finding because *O,O'*-dityrosine is a relatively minor product of $ONOO^-$ (Pennathur et al., 1999). Conversely, myeloperoxidase (MPO), and not $ONOO^-$, seems to promote *O,O'*-dityrosine formation in this model of PD (Pennathur et al., 1999). Moreover, MPO can use the NO degradation product NO_2^- to generate reactive nitrogen species (RNS) (van der Vliet et al., 1997), and studies of mice deficient in MPO demonstrate that this enzyme is one of the major sources of 3-nitrotyrosine during acute inflammation (Gaut et al., 2002). Thus, these results raise the unanticipated possibility that MPO, a heme enzyme expressed in abundance in a variety of phagocytic cells (Hampton et al., 1998), would contribute to the MPTP-induced neurodegenerative process and would represent a previously unrecognized culprit in the inflammatory-mediated oxidative insult associated with diseases such as PD. Consistent with this hypothesis, we

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show here not only that MPO is detected in affected brain areas of MPTP-injected mice and PD patients, specifically in glial cells, but also that mutant mice deficient in MPO are more resistant to MPTP-induced dopaminergic neurotoxicity. These findings indicate that MPO does participate in the MPTP neurotoxic process and suggest that inhibitors of MPO may provide protective benefit in PD.

Materials and Methods

Animals and treatment. Procedures using laboratory animals were in accordance with the National Institutes of Health (NIH) guidelines for the use of live animals and approved by the Institutional Animal Care and Use Committee of Columbia University. The mice used in this study were 10-week-old male C57BL/6J mice (Charles River Laboratories, Wilmington, MA) and MPO-deficient mice that had been backcrossed >10 times into the C57BL/6J background (Brennan et al., 1985) and their wild-type (WT) littermates, all weighing 22–25 g. For MPTP intoxication, 4–10 mice per group received four intraperitoneal injections every 2 h of MPTP-HCl (18–20 mg/kg of free base; Sigma-Aldrich, St. Louis, MO) dissolved in saline. Mice were killed from 0–7 d after the last injection, and their brains were used for morphological and biochemical analyses. Control mice received saline only. MPTP handling and safety measures were in accordance with published guidelines (Przedborski et al., 2001).

RNA extraction and reverse transcription-PCR. Total RNA was extracted from selected brain regions and at selected time points after MPTP and used for reverse transcription-PCR analysis as described previously (Wu et al., 2003). The primers used for mouse MPO and β-actin were as follows: MPO, 5′-AGGATAGGACTGGATTGCCTG-3′ (forward) and 5′-GTGGTGATGCCAGTGTTGTCA-3′ (reverse); β-actin, 5′-CTTTGATGTCACGCACGATTTC-3′ (forward) and 5′-GGGCCGCTCTAGGCACCAA-3′ (reverse). The thermal cycling conditions of the PCR were 94°C for 3 min, followed by 23–35 cycles for 20 s at 94°C, 1 min at 60°C, 1 min at 72°C, and a final extension at 72°C for 5 min. After amplification, products were separated on a 5% PAGE and quantified by a FluorChem 8800 digital image system (Alpha Innotech, San Leandro, CA). PCR products were of expected sizes, and sequences were confirmed by direct cycle sequencing.

Immunoblots. Mouse brain protein extracts from selected regions were prepared and used for Western blot analysis as described previously (Wu et al., 2003). The primary antibodies used were as follows: a rabbit polyclonal antibody raised against a 14 aa peptide representing the C terminus of the mouse MPO (NTLPKLNLTSWKET; 1:1000 dilution; generated by J.W.H.'s laboratory) and a mouse monoclonal anti-β-actin antibody (1:10,000; Sigma, St. Louis, MO). A horseradish-conjugated secondary antibody (1:500–1:25,000; Amersham Biosciences, Piscataway, NJ) and a chemiluminescent substrate (SuperSignal Ultra; Pierce, Rockford, IL) were used for detection. Bands were quantified using the FluorChem 8800.

MPO isolation and activity. The methods used to prepare brain samples and to measure MPO activity are slight modifications of those described previously by Daugherty et al. (1994). In brief, fresh mouse tissues from selected brain regions were homogenized in a 100 mM sodium phosphate buffer, pH 7.0, containing 1% (wt/vol) cetyltrimethylammonium bromide (CTAB) and centrifuged (6000 × *g*, 4°C, 10 min). Then 1 mM $CaCl_2$, $MnCl_2$, and $MgCl_2$ (final concentration) were added to each sample before being incubated overnight at 4°C with 0.3 ml of concanavalin A-Sepharose B (Sigma). The gel was then pelleted by centrifugation and washed three times with a 0.1 M sodium acetate buffer, pH 6.0, containing 0.1 M NaCl and 0.05% CTAB. Then samples were centrifuged (6000 × *g*, 5 min) to remove residual washing buffer. The glycoprotein bound to the lectin gel was then eluted by incubation with 0.15 ml elution buffer (0.5 M methyl α-D-mannoside in washing buffer) for 30 min. After the last centrifugation, final supernatants were collected and used immediately to assess MPO activity by monitoring the oxidation of tetramethylbenzidine as described previously (Andrews and Krinsky, 1982). The absorbance was read at 655 nm with a microplate reader (Bio-Rad, Hercules, CA).

Mouse MPO, glial fibrillary acidic protein, β_2 integrin MAC-1 (CD11b/CD18), neutrophil, and tyrosine hydroxylase immunohistochemistry. At selected time points after MPTP, mice were killed, and their brains were processed for immunohistochemical studies following our standard protocol for single or double immunostaining (Wu et al., 2003). The primary antibodies used were rabbit polyclonal anti-MPO (1:500; Lab Vision, Fremont, CA), rabbit polyclonal anti-glial fibrillary acidic protein (GFAP; 1:500; Chemicon, Temecula, CA), mouse monoclonal anti-MAC-1 (1:1000; Serotec, Raleigh, NC), and the monoclonal rat anti-mouse neutrophil antibody MCA771GA (1:100; Serotec). Immunostaining was visualized by 3,3′-diaminobenzidine (DAB) or fluorescein and Texas Red (Vector Laboratories, Burlingame, CA) and examined by regular or confocal microscopy. Colocalization studies were performed on doubly immunofluorescent stained sections, which were analyzed with an LSM 510 META laser-scanning microscope (Zeiss, Thornwood, NY).

For quantitative tyrosine hydroxylase (TH) immunostaining, mice were killed 7 d after MPTP. Both striatal and nigral sections (30 μm), spanning the entire extent of the structures, were incubated with a polyclonal anti-TH antibody (1:1000; Calbiochem, San Diego, CA) for 48 h at 4°C. Immunoreactivity was visualized by incubation in DAB, glucose, and glucose oxidase, and sections were counterstained with thionin. The total numbers of TH- and Nissl-positive neurons in the SNpc were counted stereologically using the optical fractionator method (West, 1993) as used previously (Tieu et al., 2003). Striatal OD of TH immunostaining, determined by the Scion (Frederick, MD) Image program, was used as an index of striatal density of TH innervation (Tieu et al., 2003). The concentration of anti-TH antibody and DAB used here and the length of time striatal sections were incubated in DAB were the same as reported previously (Tieu et al., 2003).

Human samples. All human samples were obtained from the New York Brain Bank at Columbia University (http://cumc.columbia.edu/research/equip/eq-tb_bb.htm). Procedures using this autopsy material were in accordance with the NIH guidelines for human studies and approved by the Institutional Review Board of Columbia University. Samples used in this work included the cerebellum, striatum, and ventral midbrain (for PD and controls); the caudate nucleus [for Huntington's disease (HD) and controls]; and the frontal motor cortex [for amyotrophic lateral sclerosis (ALS) or motor neuron disease and controls]. All of these cases were selected on the basis of neuropathological diagnoses using the criteria for definite PD, HD, and ALS outlined in the supplemental material (available at www.jneurosci.org). Relevant clinical and neuropathological information regarding all of the cases used here are presented in supplemental Table 2 (available at www.jneurosci.org as supplemental material). The procedures for Western blot analysis and immunohistochemistry in human tissues were identical to those described above in mouse tissues; the primary anti-MPO antibody was a rabbit anti-human MPO antibody (DakoCytomation, Carpinteria, CA) used at 1:1000 for Western blot and 1:200 for immunohistochemistry, as well as a rabbit polyclonal anti-GFAP antibody (1:10,000; DAKO, Carpinteria, CA). Visualization of the bound antibody was achieved using chromogenes SG (blue/gray) and 3-amino-9-ethylcarbazole (red) from Vector Laboratories.

MPTP metabolism. Striatal 1-methyl-4-phenylpyridinium (MPP^+) levels were determined by HPLC with UV detection (λ = 295 nm) in WT and MPO-deficient mice at 90 min after the last injection of 20 mg/kg MPTP. Striatal tissue lactate production induced by MPP^+ and synaptosomal uptake of [^{3}H]MPP^+ were performed as described previously (Wu et al., 2003). The assays were repeated three times, each time using duplicate samples.

Mass spectrometric analysis. At selected time points, anesthetized mice were perfused with ice-cold 50 mM sodium phosphate, pH 7.4, containing an antioxidant mixture made of 100 μM diethylenetriaminepentaacetic acid, 1 mM butylated hydroxytoluene, 10 mM 3-amino-1,2,4-triazole, and 1% ethanol (v/v) to minimize *ex vivo* oxidation. The ventral midbrain and cerebellum were then dissected and pulverized in liquid N_2, delipidated, dialyzed to remove low-molecular weight compounds, and hydrolyzed using HBr instead of HCl to prevent artifactual chlorination. [$^{13}C_6$]-Ring-labeled internal standards were added before hydrolysis. The amino acids were isolated using a C-18 solid-phase extraction col-

umn and subjected to derivatization and analysis by isotope dilution gas chromatography/mass spectroscopy (GC/MS) (Heinecke et al., 1999).

Detection of hypochlorous acid-modified protein. Immunochemical detection of hypochlorous (HOCl)-modified proteins was performed with the antibody HOP-1 (clone 2D10G9; dilution 1:500; provided by E. Malle, Medical University of Graz, Graz, Austria). HOP-1 is specific for HOCl-modified epitopes/proteins and does not cross-react with other oxidative modifications (Malle et al., 1995; Hazell et al., 1996). Immunostaining was visualized by using DAB, and sections were counterstained with methylgreen (Vector Laboratories).

Statistical analysis. All values are expressed as mean ± SEM. Differences among means were analyzed using one- or two-way ANOVA with time, treatment, or genotype as the independent factors. When ANOVA showed significant differences, pairwise comparisons between means were tested by Newman–Keuls *post hoc* testing. In all analyses, the null hypothesis was rejected at the $p \leq 0.05$ level.

Results

MPO is induced in the mouse ventral midbrain during MPTP-induced dopaminergic neurodegeneration

To examine the possibility that MPO is a component of the inflammatory response seen in the MPTP model of PD (Liberatore et al., 1999; Wu et al., 2002), we first assessed MPO mRNA and protein content in the ventral midbrain (i.e., brain region containing the SNpc dopaminergic neurons) over the entire active phase of neurodegeneration and gliosis provoked by this neurotoxin (Liberatore et al., 1999; Przedborski and Vila, 2001). In saline-injected control mice, the ventral midbrain contained low levels of MPO mRNA and protein (Fig. 1*A–C*). In contrast, in MPTP-injected mice, ventral midbrain levels of both MPO mRNA and protein increased in a time-dependent manner (Fig. 1*A–C*). Ventral midbrain MPO mRNA and protein expression levels peaked at 1 and 2 d after MPTP exposure, respectively (Fig. 1*C*), which is contemporaneous to the most-intense phase of SNpc dopaminergic neuronal death in this PD model (Przedborski and Vila, 2001). We next asked whether the observed changes in MPO ventral midbrain content in MPTP-injected animals paralleled an alteration of MPO enzymatic activity by monitoring oxidation of tetramethylbenzidine. Consistent with the protein data, we found that ventral midbrain MPO activity also rose during MPTP neurotoxicity in a time-dependent manner (Fig. 1*D*). In contrast, in mutant mice deficient in MPO ($MPO^{-/-}$; $n = 2$), the ventral midbrain did not show higher oxidation of tetramethylbenzidine after MPTP administration (data not shown). Unlike in the ventral midbrain, levels of MPO mRNA, proteins, and catalytic activity in the cerebellum (brain region resistant to MPTP) were unaffected by MPTP administration. However, more unexpected was the finding that no MPO alteration could be detected in the striatum (where dopaminergic fibers degenerate after MPTP administration), as illustrated by the lack of change in striatal MPO activity: saline, 14.0 ± 4.1 ($n = 7$), versus MPTP (at 2 d), 16.2 ± 1.5 ($n = 11$; $p > 0.05$). Thus, both protein levels and activity of MPO increase in the MPTP mouse model of PD, specifically in ventral midbrain where the demise of the nigrostriatal dopaminergic neurons is taking place.

MPO is expressed in reactive astrocytes after MPTP injection

To elucidate the cellular origin of MPO in the ventral midbrain of MPTP-treated mice, immunohistochemical studies were performed. In saline controls, diffuse MPO immunoreactivity was seen in the neuropil (Fig. 2*A*,*C*). In MPTP-treated mice 2 d after the last injection, ventral midbrain MPO immunostaining was stronger, especially at the level of the substantia nigra, and cells with a glial morphology appeared labeled (Fig. 2*B*,*D*). These MPO-positive cells showed punctate immunoreactivity over both the cell bodies and proximal processes (Fig. 2*D*). To corroborate the bright-field microscopy results, we performed double-immunofluorescence confocal microscopy on ventral midbrain sections from mice 2 d after MPTP. This analysis confirmed that MPO colocalized with the astrocytic marker GFAP as shown by the merged image from the two fluorochromes (Fig. 2*E–G*) and

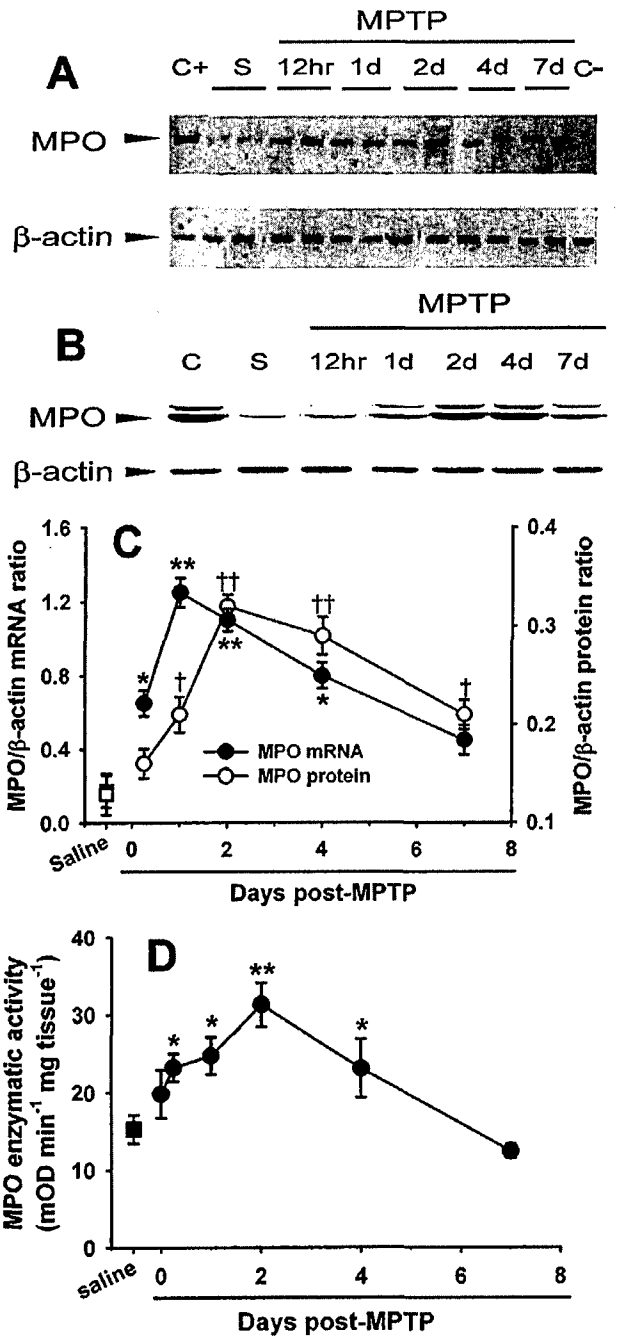

Figure 1. MPTP injections are associated with a time-dependent increase in ventral midbrain MPO mRNA (*A*, *C*), protein expression (*B*, *C*), and enzymatic activity (*D*) relative to saline injections. Data are means ± SEM for 3–11 mice per group. $^{*,\dagger}p < 0.05$, $^{**,\dagger\dagger}p < 0.01$ compared (Newman–Keuls *post hoc* test) with saline-injected control animals. S, Saline; C+, positive control (bone marrow); C−, negative control (absence of reverse transcriptase); mOD, millioptical density.

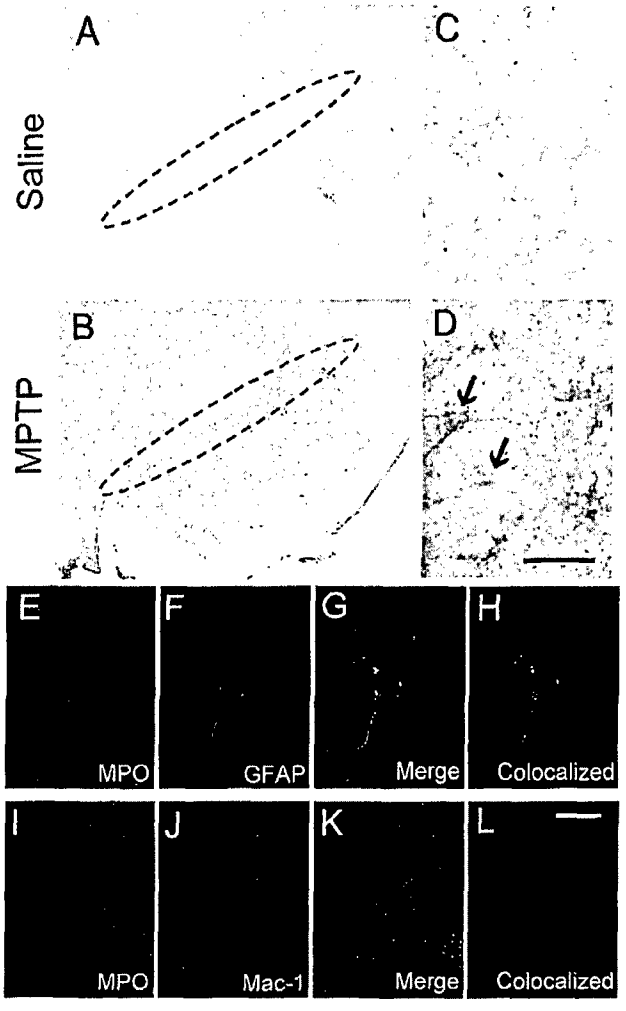

Figure 2. *A*, *C*, Immunochemical studies revealed no specific MPO immunoreactivity in the ventral midbrain of saline-injected control mice. The dashed oval delineates the SNpc. *B*, *D*, However, a dense network of fibers and scattered cell bodies positive for MPO are seen at the level of the SNpc after MPTP injections. Black arrows in *D* show the MPO-positive cellular elements. *E–H*, Confocal microscopy demonstrates that ventral midbrain MPO-positive structures (*E*, red) are also GFAP positive (*F*, green), as evidenced by the overlay of the two fluorochromes (*G*) and by the computed mask of the colocalized pixels (*H*). *I–L*, In contrast, ventral midbrain MPO-positive structures (*I*, red) are not MAC-1 positive (*J*, green), as evidenced by the overlay (*K*) and the mask of colocalized pixels (*L*). Tissue sections are from mice at 24 and 48 h after saline or MPTP injections. Scale bars: (in *D*) *A*, *B*, 250 μm; *C*, *D*, 25 μm; (in *L*) *E–L*, 10 μm.

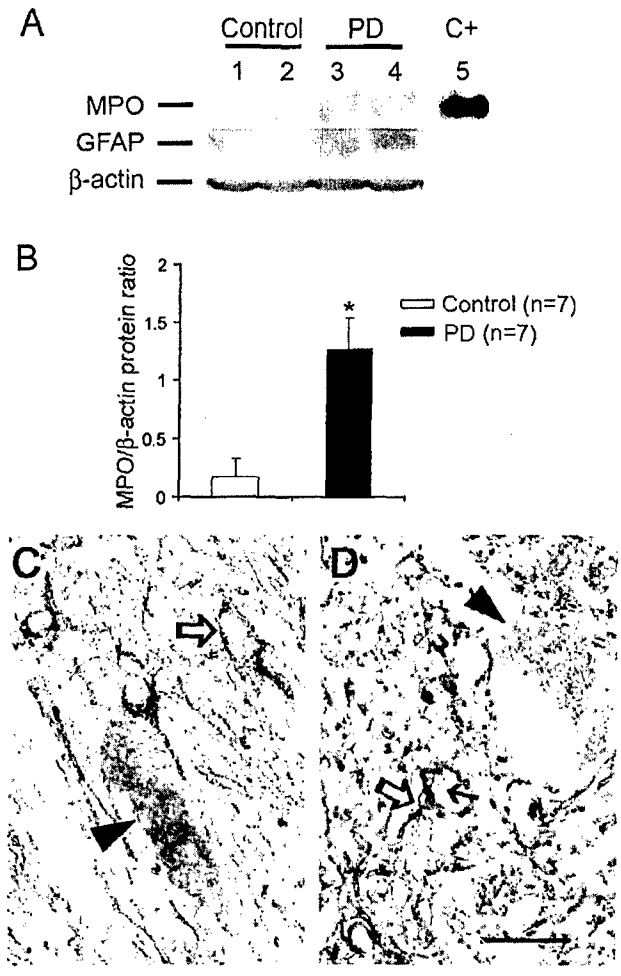

Figure 3. *A*, *B*, Ventral midbrain MPO tissue content is increased in postmortem tissue from PD patients compared with controls, as well as GFAP tissue content. C+, Positive control (purified MPO). *C*, In ventral midbrain sections, MPO (blue) is not detected in control tissues, neither in GFAP-positive cells (open arrow) nor in or around neuromelanized dopaminergic neurons (arrowhead). *D*, Conversely, MPO immunoreactivity (blue, small black arrow) is found in GFAP-positive cells (open arrow) in PD tissue but not in the rare remaining neuromelanized dopaminergic neurons (arrowhead). Scale bar, 20 μm. Data are means ± SEM for seven samples per group. *$p < 0.05$ compared with normal controls (Newman–Keuls *post hoc* test).

the computed mask of the colocalized pixels (Fig. 2*H*). Conversely, no evidence of MPO expression in microglial cells could be documented by using the same techniques (Fig. 2*I–L*). Although abundant neutrophils were seen in our mouse bone marrow preparations (positive controls) using the anti-mouse neutrophil antibody MCA771GA, none were detected within the brain parenchyma (data not shown). No noticeable cellular MPO immunoreactivity was observed in the striatum or cerebellum of either saline- or MPTP-treated mice (data not shown). These results demonstrate that MPO is primarily expressed in ventral midbrain astrocytes during the demise of dopaminergic neurons caused by MPTP.

Expression of MPO is increased in PD midbrain

To determine whether the changes in MPO observed in the MPTP mouse model of PD were present in the human condition, we assessed MPO protein levels in postmortem ventral midbrain samples from sporadic PD patients. Consistent with the mouse data, PD samples had significantly higher MPO protein contents compared with controls (Fig. 3*A*,*B*). Like in mice, there was no significant difference in MPO to β-actin ratios in the striatum (PD, 1.1 ± 0.8, vs controls, 1.4 ± 0.8; $p > 0.05$; $n = 7$) or cerebellum (PD, 0.8 ± 0.2, vs controls, 1.0 ± 0.3; $p > 0.05$; $n = 7$) between the PD and control samples. Histologically, cellular MPO immunoreactivity was not detected in the control ventral midbrain parenchyma per se (Fig. 3*C*) but only in small cells within blood vessels. However, MPO immunoreactivity was seen in ventral midbrain sections from PD patients (Fig. 3*D*), where it was identified in SNpc glial cells in the vicinity of neuromelanin-containing neurons (Fig. 3*D*). The similarity of the MPO alterations between the MPTP mice and the PD postmortem specimens strengthens the relevance of using this experimental model to study the role of MPO in the PD neurodegenerative process.

Because gliosis is a common pathological feature of many neurodegenerative diseases, we wondered whether increases in

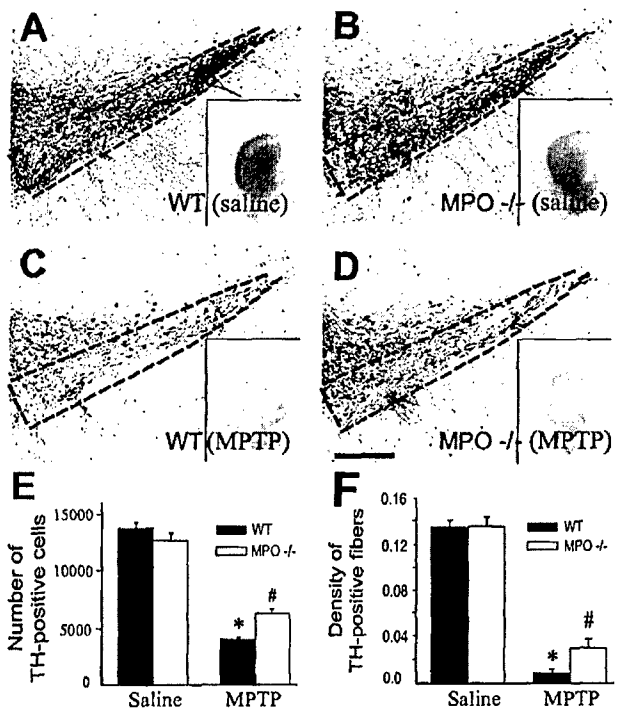

Figure 4. *A–D*, Ablation of MPO in mutant mice attenuates MPTP-induced striatal TH fibers and SNpc TH neuronal loss, as assessed 7 d after either saline or MPTP injections. *E*, *F*, Quantification of neuronal (*E*) and fiber (*F*) loss. Data are means ± SEM for four to six mice per group. $^{*}p < 0.05$ compared with saline-injected animals; $^{\#}p < 0.05$ compared with saline- and MPTP-injected MPO $^{+/+}$ mice.

the expression of MPO within areas of neurodegeneration can be found in neurodegenerative disorders other than PD. Compared with controls, the motor cortex from ALS patients did not exhibit higher GFAP or MPO values (data not shown). Conversely, we found that caudate nucleus tissues from stage 4 HD patients had higher GFAP to β-actin ratios (HD, 0.7 ± 0.1, vs controls, 0.1 ± 0.1; $p < 0.01$; $n = 3–4$) as well as MPO to β-actin ratios (HD, 0.8 ± 0.2, vs controls, 0.2 ± 0.1; $p < 0.05$; $n = 5–6$). This suggests that brain MPO expression is not specific to PD but rather generic to neurodegenerative diseases in which areas of neuronal loss are accompanied with gliosis.

MPO deficiency protects against MPTP-induced neurodegeneration

Next we compared the effects of MPTP on the nigrostriatal pathway of mutant mice deficient in MPO (MPO $^{-/-}$) and their WT littermates (MPO $^{+/+}$). Seven days after the last injection of saline or MPTP, the brains of these animals were processed for quantification of dopaminergic cell bodies in the SNpc and of projecting dopaminergic fibers in the striatum using TH immunostaining. In saline-injected MPO $^{-/-}$ and MPO $^{+/+}$ mice, stereological counts of SNpc dopaminergic neurons and striatal TH-positive OD (Fig. 4*A*,*B*,*E*,*F*) were comparable. In MPTP-injected MPO $^{+/+}$ mice, there was a ~70% loss of SNpc TH-positive neurons (Fig. 4*C*,*E*) and ~92% reduction of striatal TH OD (Fig. 4*C*,*F*) compared with saline-injected controls (Fig. 4*A*,*E*,*F*). In contrast, in MPTP-injected MPO $^{-/-}$ mice, there was only ~50% loss of SNpc TH-positive neurons (Fig. 4*D*,*E*) and ~70% reduction of striatal TH OD (Fig. 4*D*,*F*) compared with saline-injected controls (Fig. 4*B*,*E*,*F*). The TH/Nissl ratio of neuronal counts did not differ between saline- and MPTP-injected WT mice (saline, 1.68 ± 0.15, vs MPTP, 1.46 ± 0.20; $n = 5$ per group), supporting the assertion that the reduction in dopaminergic neuron numbers corresponds to an actual cell loss and not to a downregulation of TH.

To examine whether MPO ablation protects not only against structural damage but also against functional deficits caused by MPTP, we compared the levels of dopamine and its metabolites, dihydroxyphenylacetic acid and homovanillic acid, in the striatum as well as locomotor activity between MPO $^{-/-}$ and MPO $^{+/+}$ mice, after MPTP injections. Contrasting with the protection afforded by the lack of MPO on the nigrostriatal dopaminergic neurons, the loss of striatal dopamine and the deficit in motor performance caused by MPTP were as severe in MPO $^{-/-}$ as in MPO $^{+/+}$ mice (supplemental material, available at www.jneurosci.org).

MPTP metabolism

Major factors controlling MPTP neurotoxicity are its conversion in the brain to MPP $^{+}$, followed by MPP $^{+}$ entry into dopaminergic neurons and its subsequent blockade of mitochondrial respiration (Przedborski and Vila, 2001). To ascertain that the resistance of MPO $^{-/-}$ mice was not attributable to alterations in MPTP toxicokinetics, we assessed its three key neurotoxic steps (Tieu et al., 2003). Results show that striatal levels of MPP $^{+}$, striatal uptake of [^{3}H]MPP $^{+}$, and MPP $^{+}$-induced lactate production (a measure of mitochondrial function) did not differ between MPO $^{-/-}$ mice and their WT littermates (Table 1).

MPO damages ventral midbrain proteins

MPO is the only known mammalian source of HOCl at plasma concentrations of halide ion (Gaut et al., 2001). HOCl reacts with tyrosine to form 3-chlorotyrosine, a specific and stable biomarker of protein damage by MPO (Heinecke et al., 1999). To determine whether MPTP promotes oxidative damage to brain proteins, we used isotope dilution GC/MS (Heinecke et al., 1999), a sensitive and specific method, to quantify 3-chlorotyrosine levels in samples from eight saline-injected controls and eight MPTP-injected mice 24 h after injection. We compared levels of 3-chlorotyrosine in the ventral midbrain and cerebellum. In MPTP-treated mice, 3-chlorotyrosine levels in the ventral midbrain were markedly increased ($p < 0.05$) compared with saline-injected controls: MPTP, 30.8 ± 5.7 nmol of 3-chlorotyrosine per molar of tyrosine ($n = 8$) versus saline controls, 4.8 ± 2.1 nmol of 3-chlorotyrosine per molar of tyrosine ($n = 8$). 3-Chlorotyrosine was undetectable in the cerebellum of mice injected with either saline or MPTP. In contrast, in MPTP-treated MPO $^{-/-}$ mice ($n = 3$), ventral midbrain 3-chlorotyrosine was undetectable. The identification of chlorinated tyrosine in tissues therefore supports the hypothesis that reactive intermediates produced by MPO damage brain proteins in MPTP-intoxicated mice.

To localize MPO-damaged proteins, tissue sections were immunostained with HOP-1, a mouse antibody that specifically recognizes HOCl-modified proteins (Malle et al., 1995); the chlorotyrosine antibody was not available to us. Intense HOP-1 immunoreactivity was observed in the SNpc of MPTP-injected mice (Fig. 5*A–C*). HOP-1-positive material was seen in the neuropil within beaded-appearing fibers and in cells with both neuronal and non-neuronal morphology within vesicular elements (Fig. 5*A–C*). No HOP-1 immunostaining was detected in the SNpc of saline-injected mice or MPTP-injected MPO $^{-/-}$ mice (data not shown).

Table 1. Striatal MPTP metabolism in MPO-deficient mice

	MPP^+ level (μg/g striatum)	MPP^+ uptake (IC_{50}, nM)	MPP^+-induced lactate (μM/100 mg protein)
$MPO^{+/+}$ mice	4.46 ± 0.24	113.7 ± 1.2	57.6 ± 7.5
$MPO^{-/-}$ mice	5.54 ± 0.71	114.3 ± 1.7	66.8 ± 4.4

Striatal MPP^+ levels in WT ($MPO^{+/+}$) and MPO-deficient mice ($MPO^{-/-}$) were determined 90 min after the last injection of MPTP (20 mg/kg). Values are means ± SEM of either six mice per group (MPP^+ level) or three independent experiments each performed in duplicate ([^{3}H]MPP^+ uptake and lactate level). None of the presented values differ significantly ($p > 0.05$) between $MPO^{+/+}$ and $MPO^{-/-}$ mice.

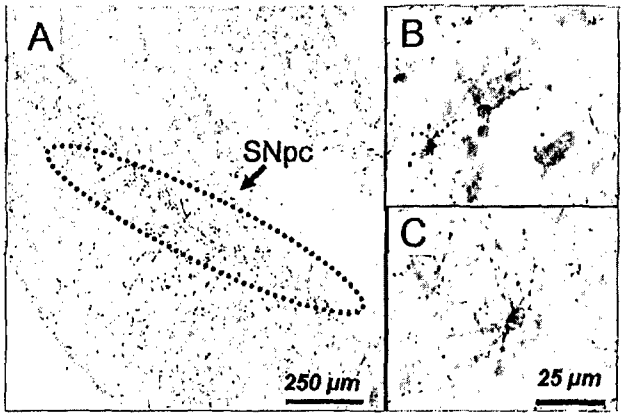

Figure 5. Immunohistochemical localization of HOCl-modified proteins with the HOP-1 antibody in ventral midbrain sections. Twenty-four hours after MPTP injections, HOP-1-positive immunoreactive material is seen mainly at the level of the SNpc (*A*) and within or around cellular elements (*B*, *C*). Scale bars: *A*, 250 μm; *B*, *C*, 25 μm.

Discussion

The present study shows that the level of MPO expression increases markedly in diseased SNpc from both mice exposed to MPTP (Figs. 1, 2) and human PD (Fig. 3). This work also demonstrates that changes of MPO protein content and enzymatic activity in MPTP-intoxicated mice parallel (Fig. 1) the degeneration of SNpc dopaminergic neurons (Przedborski and Vila, 2001). Moreover, MPO is found primarily in SNpc-reactive astrocytes (Figs. 2, 3), which are major cellular components of the PD- and MPTP-associated inflammatory response (Przedborski and Goldman, 2004). Conversely, we failed to detect any of the well established cellular sources of MPO (neutrophils, monocytes, or macrophages) (Hampton et al., 1998) within the ventral midbrain parenchyma of PD patients and MPTP-injected mice. The presence of MPO in damaged SNpc thus appears to derive essentially from a resident, not a blood-borne, inflammatory response associated with the degeneration of dopaminergic neurons. Based on assessments performed in two other neurodegenerative diseases, namely HD and ALS, it appears that MPO upregulation in the brain is not pathognomonic of PD. Instead, we believe that the occurrence of MPO in diseased brains is likely indicative of a disease process associated with chronic gliosis rather than a particular etiology. That said, our results are surprising because phagocytic white blood cells are generally believed to be the only cellular sources of MPO. However, neuronal expression of MPO is also increased in Alzheimer's disease (Green et al., 2004), raising the possibility that this enzyme may contribute to oxidative damage in a variety of chronic neurodegenerative disorders.

Contrary to ventral midbrain, the striatum, which is also a site of a strong inflammatory reaction after MPTP administration and sometimes in PD, did not show any alteration in MPO expression or enzymatic activity as illustrated in Figure 3. Remarkably, detectable changes in iNOS expression and enzymatic activity are also confined to ventral midbrains of MPTP-injected mice (Liberatore et al., 1999), whereas activation of NADPH oxidase is observed in both ventral midbrains and striata of these animals (Wu et al., 2003). Collectively, these observations suggest that the molecular composition of the inflammatory response to injury may be, to a certain extent, regionally specific. Supporting this view is the finding that a stereotaxic injection of 5 μg/μl bacterial endotoxin lipopolysaccharide into the hippocampus or cortex of adult rats produces no apparent neuronal loss, whereas an identical administration into the substantia nigra dramatically reduces the number of neurons (Kim et al., 2000). Although this distinct regional susceptibility has been linked to differences in microglial densities, its molecular basis might well be related to differences in the quantity or variety of the inflammatory mediators produced.

After MPTP injections, mutant mice deficient in MPO showed more spared SNpc dopaminergic neurons and striatal dopaminergic fibers than their WT littermates (Fig. 4). We also found that the lack of MPO did not alter key aspects of MPTP toxicokinetics (Table 1). Together, these findings indicate that MPO contributes to the pathogenic cascade of deleterious events responsible for the demise of dopaminergic neurons in the MPTP model and perhaps in PD as well. Surprisingly, although alterations in MPO protein and enzymatic activity were only detected in the ventral midbrain (Fig. 1), both cell bodies and fibers of nigrostriatal dopaminergic neurons were preserved in MPTP-injected $MPO^{-/-}$ mice (Fig. 4). This observation implies that an entire neuron may be salvaged by mitigating deleterious factors that specifically injure cell bodies and that nigrostriatal dopaminergic neurons are not degenerating solely via a dying-back process, as one may have thought based on previous observations (Herkenham et al., 1991; Wu et al., 2003).

The relative resistance of dopaminergic neurons to MPTP-induced neurotoxicity in $MPO^{-/-}$ mice was, however, not accompanied by a preservation of striatal dopamine levels or attenuation of motor deficits caused by this parkinsonian neurotoxin (supplemental material, available at www.jneurosci.org). This discrepancy may be explained by the fact that TH (the rate-limiting enzyme in the synthesis of dopamine) can be inactivated by injury, such as that inflicted by MPTP (Ara et al., 1998). It is thus conceivable that although ablation of MPO attenuates the loss of TH protein (as evidenced by immunostaining), this beneficial effect may not be enough to prevent the loss of TH catalytic activity (as evidenced by the dopamine levels). Targeting MPO alone may thus suffice to provide observable structural, but not functional, neuroprotection in this experimental model of PD. Accordingly, optimal therapeutic interventions for PD may rely on the combination of strategies capable of providing structural protection such as MPO inhibition, with other strategies capable of protecting/stimulating dopaminergic function. Yet, given the relentless nature of PD, it can be surmised that the death signal in this illness may not be as harsh as that provoked by MPTP. Therefore, whether MPO inhibition in PD can succeed, not only in slowing neuronal death but also in sustaining dopamine synthesis, is a possibility that should not readily be excluded.

As to how MPO neurotoxic actions on dopaminergic neurons are mediated, two distinct and not mutually exclusive mechanisms may be invoked. First and foremost, MPO is known for its production of cytotoxic reactive oxygen species and RNS (Harrison and Schultz, 1976; Eiserich et al., 1996; Hampton et al., 1998).

Therefore, neurons located in the vicinity of MPO-containing cells may have their plasma membrane proteins and lipids subjected to the deleterious effects of MPO-derived oxidants such as HOCl. In keeping with this scenario, we found high levels of 3-chlorotyrosine, a specific oxidative modification of tyrosine residues mediated by HOCl in the MPTP-susceptible brain region, the ventral midbrain. Also supporting the oxidative role of MPO in the MPTP model is our immunohistochemical demonstration of HOCl-modified protein in the ventral midbrain of intoxicated mice (Fig. 5*A–C*). Aside from this oxidative effect, MPO can be secreted and bind CD11b/CD18 integrins to the cell surface (Lau et al., 2005). In the case of neutrophils, ligation of CD11b/CD18 by MPO stimulates signaling pathways implicated in the activation of these cells (Lau et al., 2005). Because brain microglia do express CD11b/CD18 integrins and seem to participate in the neurodegenerative process in the MPTP model and in PD, this cytokine-like effect of MPO may represent an additional mechanism by which dopaminergic neurons are affected by this enzyme.

As raised previously (Wu et al., 2003), a key issue is the selective damage to dopaminergic neurons observed during inflammation in MPTP-treated mice and humans suffering from PD. Many lines of evidence suggest that dopaminergic neurons are particularly vulnerable to oxidative stress compared with the other cells in the brain (Dauer and Przedborski, 2003). Alternatively, it is likely that in the MPTP model and in PD, the magnitude of the inflammatory response and resulting oxidative stress is mild and only inflicts sublethal lesions. Thus, inflammation-mediated oxidative stress would succeed in killing only neurons already compromised, as dopaminergic neurons probably are in PD and after MPTP injections.

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